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# The production of biogenic gases in the marine environment

A thesis presented for the degree of Doctor of Philosophy  
at the Open University

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2007

Scottish Association for Marine Science  
UHI Millennium Institute



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## Abstract

The biogenic trace gases dimethylsulphide (DMS) and methane play a major role in the Earth's climate and atmospheric chemistry. DMS makes a significant negative contribution to radiative forcing and methane is an important greenhouse gas. The marine environment is an important source of both DMS and methane and this thesis investigated the interactions between methylated sulphur compounds and methanogenesis within micro-environments in the upper-water column.

The production of DMS is closely associated with dimethylsulphoniopropionate (DMSP) and dimethylsulfoxide (DMSO) although comparatively little is known about the dynamics of DMSO in the marine environment. This study analysed the production of DMSO by a range of marine phytoplankton species in comparison with DMSP. Algal concentrations of DMSO were taxon-dependent and an average DMSPp:DMSOp ratio of 4.85 was calculated for the phytoplankton species analysed. The consistent presence of DMSO at ~20 % of total intracellular methylated sulphur (DMSP + DMSO) suggests it is an important algal constituent. The fate of DMSP and DMSO produced by algal cells was analysed in a series of grazing experiments with the copepods *Temora longicornis* and *Acartis clausi*, and the dinoflagellate *Scrippsiella trochoidea*. The relative importance of assimilation, sloppy feeding, DMS production and excretion of faecal pellets as sinks for algal-DMSP was assessed.

In comparison to the ambient marine environment, both algal cells and copepod faecal pellets were identified as micro-habitats of elevated DMSP and DMSO concentrations. Millimolar concentrations of DMSP were measured in algal cells and micromolar



concentrations of DMSP were recorded in copepod faecal pellets. These hotspots of DMSP and DMSO may be associated with chemical processes that differ from bulk seawater characteristics, such as the transformation of DMSO as revealed in coastal pelagic particulate material. Furthermore, oxygen depletion at the micro-scale could facilitate the presence of anaerobic bacteria, or anaerobic microbial activity, in the predominantly aerobic pelagic environment. The occurrence of methanogenic Archaea specifically associated with copepod faecal pellets from mono-species cultures of copepods, environmental samples and total pelagic particulates was investigated using 16S rRNA gene libraries. Clusters of sequences closely related to *Methanogenium*, *Methanobacterium* and *Methanolobus* were recovered. This work revealed that CO<sub>2</sub>-reducing methanogens can exist in the pelagic environment where it was previously assumed methylotrophic methanogenesis dominated.

This work demonstrated that the methylated sulphur compounds represent a metabolic substrate for methanogens in the upper water column. The addition of DMSP, DMSO and DMS to samples collected from the upper water column stimulated methanogenesis when incubated under anaerobic conditions. The addition of inhibitors suggest that other anaerobic microbes *e.g.* sulphate-reducing bacteria, may play a key role in this process. The potential for DMSP and DMSO to serve as precursors for methane, a potent greenhouse gas, as well as DMS, has important implications when considering the emissions of these compounds from the marine environment and their role in the Earth's climate.

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## Units, chemical symbols and abbreviations

$\mu\text{l}$	microlitre = $1 \times 10^{-6}$ litre
ml	millilitre = $1 \times 10^{-3}$ litre
$\text{dm}^{-3}$	decimetre = 1 litre
$\mu\text{m}$	micrometre = $1 \times 10^{-6}$ metre
pg	picogram = $1 \times 10^{-12}$ grams
ng	nanogram = $1 \times 10^{-9}$ grams
$\mu\text{g}$	microgram = $1 \times 10^{-6}$ grams
mg	milligram = $1 \times 10^{-3}$ grams
Tg	Teragram = $1 \times 10^{12}$ grams
Gt	Gigatonne = $1 \times 10^{15}$ grams = petagram
pmol	picomolar = $1 \times 10^{-12}$ mols
nmol	nanomolar = $1 \times 10^{-9}$ mols
$\mu\text{mol}$	micromolar = $1 \times 10^{-6}$ mols
mmol	millimolar = $1 \times 10^{-3}$ mols
M	molar
ppmv	parts per million by volume = $\mu\text{mol mol}^{-1}$
ppbv	parts per billion by volume = $\text{nmol mol}^{-1}$
$\lambda$	Wavelength
$\Delta G^{\circ}$	Standard free energy change
$\sigma$	Density of seawater determined from <i>in situ</i> temperature and salinity
AMT	Atlantic Meridional Transect
ANOVA	Analysis of Variance
ATP	Adenosine 5'-triphosphate
C	Carbon
Chla	Chlorophyll a
CLAW	Charlson, Lovelock, Andreae and Watson hypothesis
$\text{CH}_4$	Methane
Cl	Chlorine
ClO	Chlorine monoxide
CO	Carbon monoxide
$\text{CO}_2$	Carbon dioxide
CoM	CoenzymeM
DGGE	Denaturing Gradient Gel Electrophoresis
DML	Dunstaffnage Marine Laboratory
DMS	Dimethylsulphide
DMSP	Dimethylsulphoniopropionate
DMPT	Dimethylsulphoniopropionate
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleotides
DOM	Dissolved Organic Matter
DOC	Dissolved Organic Carbon

EDTA	ethylenediamine tetraacetic acid
Eh	Redox potential
FMN	Flavin Mononucleotide
FID	Flame Ionization Detector
FPD	Flame Photometric Detector
GBT	Glycine betaine
GC	gas chromatography
HCl	Hydrochloric acid
H <sub>2</sub>	Hydrogen
H <sub>2</sub> SO <sub>4</sub>	Sulfuric acid
IO	Iodine monoxide
IPA	Isopropyl alcohol
IPCC	Intergovernmental Panel on Climate Change
IPTG	Isopropyl β-D-1-thiogalactopyranoside
MgCl <sub>2</sub>	Magnesium Chloride
MSNA	methane sulphinic acid
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NaPh	Sodium Phosphate
NIO	National Institute of Oceanography
NO <sub>2</sub>	Nitrogen dioxide
NO <sub>3</sub> <sup>-</sup>	Nitrate
N <sub>2</sub> O	Nitrous oxide
NSS	Non sea salt
OFN	Oxygen-free Nitrogen
OH	Hydroxyl radical
OTU	Operational Taxonomic Unit
O <sub>2</sub>	Oxygen
O <sub>3</sub>	Ozone
PCR	Polymerase Chain Reaction
POC	Particulate Organic Carbon
PTFE	polytetrafluoroethylene
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
<i>RV</i>	Research Vessel
SDS	Sodium Dodecyl Solution
SRB	Sulphate Reducing Bacteria
SO <sub>2</sub>	Sulphur dioxide
SO <sub>4</sub> <sup>2-</sup>	Sulphate
<i>Taq</i>	<i>Thermus aquaticus</i> polymerase
TE	Tris-EDTA
UV	Ultraviolet
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

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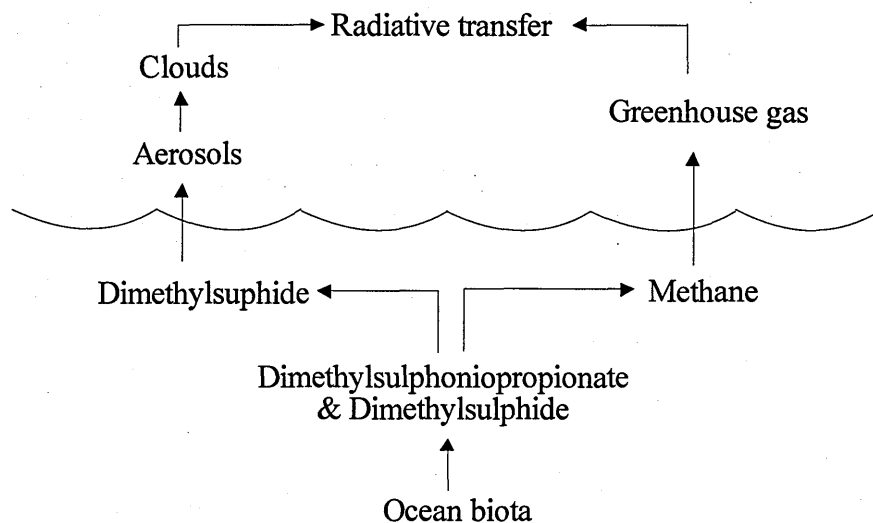
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To my wonderful family: Mum & Dad; Jay, Ana, Jason & Andrew; Jenny, Tony, Stephanie & Aimee; Maz, Nick & Neve; Beth & Paul, and Grandma Wilson. Thank you for helping me a keeping a focus on life whilst I competed the PhD and a special thanks to my siblings for taking the pressure off me to settle down and lead a normal life.

To John, Tom, Michael, Alex and Tim, who introduced me to the Scottish hills and road cycling. Thank you for being a constant source of humour, encouragement and fatigue. Reel this one in.

## Chapter 1. Introduction

The oceans are an intricate component of the Earth's climate system. A key role is played by the formation and emission of aerosol precursors and greenhouse gases in the upper water column, which affect the Earth's radiative balance. This thesis looks at two important compounds which influence the Earth's climate: dimethylsulphide (DMS) and methane (Fig. 1.1). The first section of this Chapter outlines the production and removal processes for DMS and its main precursor dimethylsulphoniopropionate (DMSP) in the upper ocean. The role of DMS in the Earth's climate is then introduced with specific reference to climatic feedback mechanisms. Then methane and the micro-organisms responsible for its production and oxidation are described, followed by an account of the distribution of methane in the surface oceans. The aims and objectives of the project are stated at the end of the Chapter.



**Figure 1.1** Diagram highlighting the connection between ocean biota and climate.

## 1.1 Marine biogenic sulphur

### 1.1.1 Cellular sulphur & DMSP

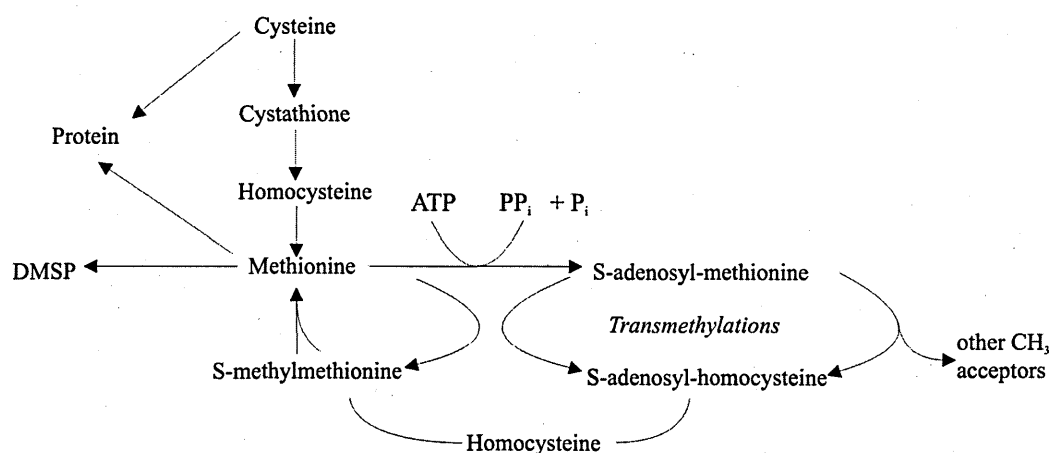
Sulphur is an essential nutrient element, found in numerous proteins, co-enzymes, vitamins and electron carrier systems crucial to cellular metabolism. Within cells, sulphur is abundant in the amino acids cysteine and methionine and it is structurally important in cell walls, photosynthetic membranes and connective tissues (Zehnder and Zinder, 1980).

Organic sulphur is typically acquired through the reduction of sulphate, but may also include thiosulphates, polythionates and elemental sulphur (Le Faou et al., 1990). Cellular sulphur also forms part of the compatible solute dimethylsulphoniopropionate (DMSP).

Major DMSP synthesis is confined to a few taxonomic groups of marine micro- and macro-algae. Observations of DMSP production in higher plants is rare and generally only include marine salt-tolerant species such as *Spartina* sp. (Dacey et al., 1987), the coastal strand plant *Wollastonia biflora* (Storey et al., 1993) and the non-marine exception, sugarcane (Paquet et al., 1994). The biosynthesis of DMSP involves the assimilation of sulphate and subsequent reduction to sulphide (Stefels, 2000) (Fig. 1.2). Free sulphide is then incorporated within *O*-acetylserine to form cysteine and acetate. Cysteine is important for cellular protein synthesis and the *de novo* production of methionine. Methionine is partly incorporated into protein, but the major pathway for methionine metabolism is the utilization of its methyl group in transmethylation reactions via *S*-adenosylmethionine (Giovanelli, 1987). In addition to its incorporation into proteins, methionine may also be used to produce DMSP (Fig. 1.2).



With regards to plant compounds, the term ‘compatible solute’ refers to the low molecular mass organic solutes produced by algae to maintain intracellular osmotic pressure at a concentration greater than the ambient environment. The ‘compatible’ status of these organic solutes reflects the fact that they do not perturb other cellular constituents, even when the solutes are at high concentration (Brown and Simpson, 1972). Many different compounds serve as organic compatible solutes, as listed in Welsh (2000), and include small carbohydrates including sugars (*e.g.* trehalose), polyols (*e.g.* glycerol); amino acids (*e.g.* glycine); glycine betaine and methylsulphonium solutes including DMSP.



**Figure 1.2** Synthesis of DMSP from cysteine by algal cells. From Stefels (2000).

In addition to their osmotic function, compatible solutes are also engaged in other cellular activity, and several physiological roles, termed ‘secondary functions’, have been ascribed to DMSP within phytoplankton cells:

- An antioxidant activity has been proposed for DMSP in marine algae whereby DMSP and the related compounds DMS, acrylate, dimethylsulphoxide (DMSO) and methanesulfinic acid, scavenge hydroxyl radicals and other reactive oxygen species (Sunda et al., 2002). In support of this hypothesis, a number of oxidative stressors including solar ultraviolet radiation, and iron limitation substantially increased cellular DMSP levels (Sunda et al., 2002; Slezak and Herndl, 2003). The possible role of

DMSP as a cellular free radical scavenger was supported by the increased concentrations under low-iron conditions in the Peruvian upwelling system, as well as by the positive correlations observed between these compounds and the known antioxidant  $\beta$ -carotene (Riseman and DiTullio, 2004).

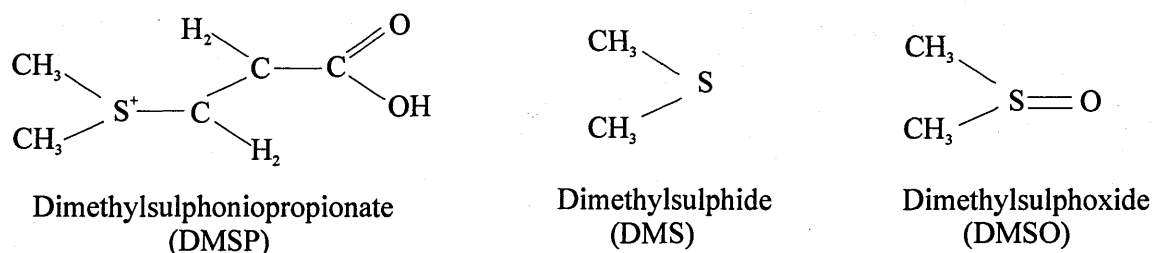
- DMSP also has protein cryoprotective properties and increased concentrations are suggested to reduce the cells freezing point (Kirst et al., 1991). Karsten et al. (1996) found DMSP stabilized enzymatic activity in the macroalgae *Acrosiphonia artca* at -2 °C and in addition at high ambient DMSP concentrations, enzyme activity increased to 165 % of the control values.
- DMSP may have a role as a grazing deterrent in algae (discussed in Chapter 4), whereby the mechanical ingestion of cells brings DMSP into contact with DMSP-lyase enzyme, and the resulting products, DMS and acrylate deter grazers e.g. protozoans (Wolfe and Steinke, 1996).
- Finally, DMSP is also thought to help dissipate excess reduced sulphur within the cell (Stefels, 2000). The concentrations of precursory amino acids cysteine and methionine are maintained at low equilibrium level of ~10  $\mu$ mol and it is important for cells to have a buffering mechanism to regulate these levels when the influx of sulphur exceeds the cell's conversion capacity into amino acids and other sulphur-containing compounds (Giovanelli, 1990). The continued production of DMSP may therefore help to keep cysteine and methionine at low concentrations preventing possible feedback mechanisms to occur which may decrease the cells capacity to incorporate nitrogen (Stefels, 2000).

These secondary functions suggest that DMSP concentrations can vary between algal individuals for reasons that are unrelated to osmotic stress. This could explain why the

cellular concentration of DMSP in phytoplankton is closely related to algal-taxonomy. The Dinophyceae (dinoflagellates) and Prymnesiophyceae (including coccolithophorids) produce higher concentrations of DMSP, whilst members of the Bacillariophyceae (diatoms) and Chrysophyceae produce modest quantities (Keller et al., 1989). DMSP can accumulate to high internal cellular concentrations, with cystolic values for major DMSP-producing algal groups ranging from 50-400 mmol cell<sup>-1</sup> (Yoch, 2002). In the major-DMSP producing algae it is estimated that over 50 % of cellular organic sulphur (Matrai and Keller, 1993), and approximately 10 % of cellular carbon (Matrai and Keller, 1994) may be in the form of DMSP. Therefore, within the marine environment, during instances of increased algal biomass such as phytoplankton blooms, DMSP represents a significant source of carbon and sulphur within the ecosystem.

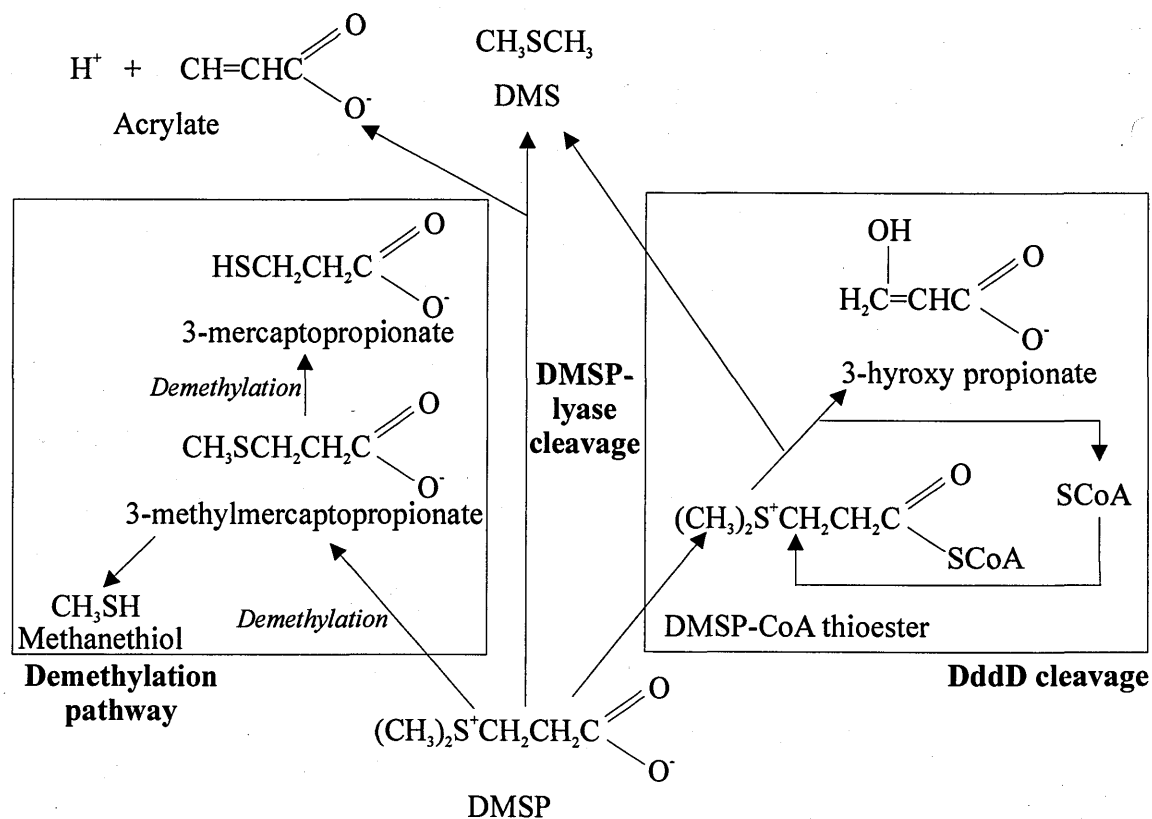
### **1.1.2 The degradation of DMSP to DMS**

Until the early 1970's, it was unclear which of the volatile sulphur compounds was responsible for closing the global sulphur budget and transferring sulphur from the sea to the air. Possible contenders included carbonyl sulphide, carbonyl disulphide and also hydrogen sulphide (Nguyen 1983). However concentrations of these compounds in seawater were insufficient to sustain the global sulphur budget. In 1972, James Lovelock proposed that dimethylsulphide (DMS) (Fig. 1.3) was the most abundant natural volatile sulphur compound in seawater and therefore the most likely source for atmospheric sulphur.



**Figure 1.3** Structural formulas of the methylated sulphur compounds DMSP, DMS & DMSO.

In the marine environment DMS is predominantly derived from the breakdown of DMSP. This occurs due to the catalysis by DMSP-lyase enzyme and is also referred to as the ‘cleavage pathway’ (Kiene, 1999) (Fig. 1.4). DMSP-lyase activity has been tentatively identified in certain bacterioplankton (Wolfe, 1996; Gonzalez et al., 1999) and phytoplankton (Steinke et al., 1998). An alternative degradation pathway for DMSP also exists which does not involve the formation of DMS (Kiene and Taylor, 1988). This is referred to as the ‘demethylation pathway’ and consists of an initial demethylation of DMSP to form 3-methiolpropionate (MMPA) (Fig. 1.4). MMPA can undergo an additional demethylation step to produce 3-mercaptopropionate (3-MPA) or yield methanethiol (Kiene and Taylor, 1988). More recently, a new pathway for DMS production from DMSP has been proposed by Dodd et al.(2006), referred to as the DddD cleavage (Fig. 1.4). The authors propose that DMSP is initially modified by the addition of acyl-coenzyme A and the DMSP-CoA thioester is subsequently catabolized by one or more steps to DMS plus 3-hydroxypropionate (Dodd et al., 2006).



**Figure 1.4** Diagram highlighting the three known DMSP degradation pathways. From Dodd et al. (2006).

Factors controlling the relative proportion of DMSP that is degraded via the cleavage or demethylation pathway are still being elucidated, but are considered pivotal to the production of DMS in the marine environment (Kiene and Service, 1991; Kiene, 1996). It has been hypothesised that bacteria exert a level of control on DMSP dynamics by preferentially degrading DMSP via demethylation when dissolved DMSP concentrations are low relative to sulphur demand (Kiene et al., 2000). This has a detrimental effect on levels of ambient DMS because none of the derivatives are readily converted to DMS (Taylor and Gilchrist, 1991). The assimilatory metabolism of DMSP is therefore hypothesised to be the proximate control on DMS formation in the upper ocean (Kiene et al., 2000).

### 1.1.3 DMSP and DMS in the marine environment

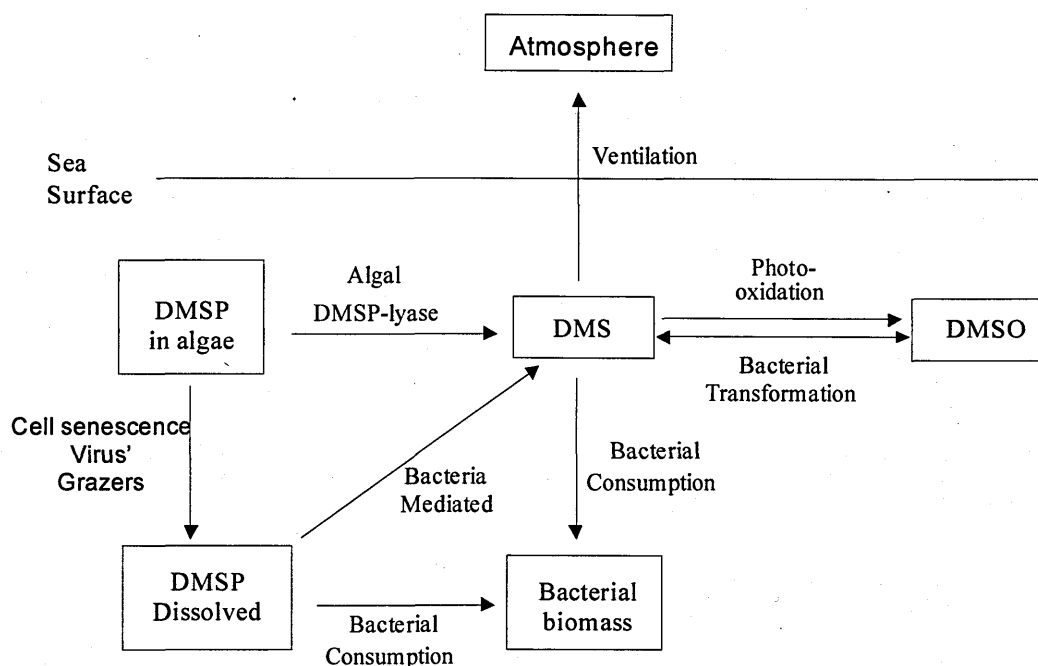
The release of DMSP from algal cells to the ambient environment, as highlighted in Figure 1.5, can occur through a number of mechanisms. These include:

- Algal senescence such as observed at the end of a phytoplankton growth cycle. During cell senescence in a laboratory culture of *Phaeocystis* sp. almost 73 % of cellular DMSP was recovered as DMS (Stefels 1993). Premature senescence may also be induced by nutrient-limitation and other stress factors and these may also indirectly release cellular DMSP (Keller and Korjeff-Bellows, 1996; Slezak and Herndl, 2003).
- Viral lysis of algal cells can also influence nutrient cycling (Suttle, 2005). Viral lysis of non-axenic *Phaeocystis pouchetti* cultures has been found to cause a 8-14 fold increase in concentrations of DMSPd after 48 hours (Malin 1998). A similar result was also demonstrated in viral-infected cultures of *Micromonas pusilla* cultures (Hill 1998). However, during field-based research no significant correlation was observed between viral abundance and DMS/DMSPd concentrations or nutrient-enriched mesocosms in a Norwegian fjord (Wilson et al., 1998).
- The release of DMSP into solution and the production of DMS can be accelerated during grazing by both micro- (Wolfe et al., 1994) and macro-zooplankton (Dacey and Wakeham, 1986) (discussed further in Chapter 4). The increase in the levels of DMSPd observed during grazing by *Oxyhris marina* on *Emiliania huxleyi*, was influenced by *O. marina* cell density (Wolfe et al., 1994). At lower prey densities (150 cells cm<sup>-3</sup>) approximately 75 % of DMSPp was released into solution.

Once released to the ambient environment, DMSP is rapidly metabolised by bacteria.

DMSP can supply 1-15 % of carbon and virtually all sulphur demand of the

bacterioplankton (Kiene and Linn, 2000; Simo et al., 2002). Turnover rates are comparable to those of other low molecular weight compounds *e.g.* amino acids, glucose (Zubkov et al., 2002). However it is not known whether DMSP is metabolised by all marine bacterioplankton or restricted to a few phylotypes with defined ecological niches (Vila et al., 2004). It has been suggested that a prominent role in DMSP turnover is played by a few bacterial groups including *Roseobacter* clades, *Cytophaga-Flavobacterium* and SAR11 (Gonzalez et al., 1999; Zubkov et al., 2002; Vila et al., 2004).



**Figure 1.5** Classical diagram of the DMS(P) biogeochemical cycle in the upper water column.

An under-stated component of the biogenic sulphur cycle is dimethylsulphoxide (DMSO) (Fig. 1.3). DMSO is ubiquitous in seawater where it contributes to the pool of dissolved methylated sulphur compounds, alongside DMS and DMSPd (Simo and Vila-Costa, 2006). Much of the early microbiological research on DMSO focused on its role as an electron

acceptor during anaerobic respiration (McCrindle et al., 2005). It has also been demonstrated that in addition to anaerobic respiration, several aerobic bacteria are also able to oxidise DMS to DMSO and this has been demonstrated for certain strains of the *Roseobacter* clade of the Proteobacteria  $\alpha$ -subclass (Gonzalez et al., 1999). The conversion of DMS to DMSO was recently quantified through the addition of  $^{35}\text{S}$ -DMS to incubations of natural seawater (del Valle et al., 2007). The authors found that DMSO and sulphate accounted for 81-93 % of the non volatile sulphur products. DMSO also represents a carbon and sulphur source for marine microbes and the consumption of DMSO has been demonstrated for the methylotroph *Hyphomicrobium* sp. under aerobic conditions (de Bont et al., 1981; Suylen et al., 1986). Representations of the biogeochemical DMS(P) cycle (e.g. Fig. 1.5) typically show DMSO as an ambient compound in the seawater, and produced from DMS either by photo-chemical or microbial oxidation (see Section 1.1.4). However, neither the production or removal pathways for DMSO have been subject to the same level of scrutiny as applied to DMSP and DMS. Furthermore, there is increasing evidence for a particulate pool of DMSO which is associated with phytoplankton (Simo et al., 1998; Hatton and Wilson, 2006; Simo and Vila-Costa, 2006).

#### 1.1.4 The fate of DMS in the marine environment

Concentrations of DMS in the surface oceans range between 0.5-10 nmol dm<sup>-3</sup> with a global average of ~4 nmol dm<sup>-3</sup> (Kettle et al., 1999). The narrow range of concentrations in the marine environment is considered to be indicative of the loss processes occurring in tight coupling with production. However, whilst the formation of DMS is considered to be an almost exclusively biological process, its loss and degradation occur through a combination of biological and physico-chemical pathways. The major processes that



remove DMS from the mixed layer include: microbial metabolism; oxidation; and ventilation.

The microbial consumption of DMS is typically considered to be the predominant factor in determining surface concentrations. An increase in DMS concentrations is thought to occur when consumption is uncoupled from production (Zubkov et al., 2004). Instances of this include intensive grazing events and phytoplankton senescence (Kwint and Kramer, 1995; Nguyen et al., 1998) and the early exponential increase in phytoplankton blooms (van Duyl et al., 1998; Zubkov et al., 2004). The microbial metabolism of DMS may be due to obligate methylotrophs or generalists that can use a variety of carbon sources (Visscher and Taylor, 1994; de Zwart and Kuenen, 1997; Gonzalez et al., 1999; Hoefft et al., 2000).

There are also interesting discrepancies between the microbial utilization of DMS and DMSP (Fig. 1.3). Turnover times for DMS (0.5-4 days) in the marine environment are lower (Kieber et al., 1996; Zubkov et al., 2002) compared to DMSP (0.5-1 day) (Kiene and Linn, 2000; Zubkov et al., 2002; Malmstrom et al., 2004). DMS also represents a supplementary carbon and electron source for bacterioplankton. However, less than 2 % of metabolised [ $^{35}\text{S}$ ]-DMS was retained by bacterial cells, possibly as a reduced sulphur source for protein synthesis (Kiene et al., 1999). The remainder was transformed into dissolved non-volatile products, which is likely to include DMSO and sulphate (del Valle et al., 2007).

The oxidation of DMS may be photo-chemical (Kieber et al., 1996) or microbially-mediated (Taylor and Kiene, 1989). The microbial oxidation of DMS occurs under both anaerobic (Zeyer et al., 1987) and aerobic conditions (Fuse et al., 1998). During

incubations of coastal and open ocean seawater approximately 70 % of DMS added was converted to DMSO (Vila-Costa et al., 2006). The photo-oxidation of DMS to DMSO was first implied by Brimblecombe and Shooter (1986) and since then estimates of DMS removal via photo-oxidation are <37 % in the North Sea (Hatton, 2002) and 14 % in the equatorial Pacific (Kieber et al., 1996).

The flux of DMS from the ocean to the atmosphere is a minor pathway in its overall budget in the upper ocean and it is typically assumed that ~1 % of gross DMSP production will be released into the atmosphere by the sea-air DMS flux (Simó, 2001). During a coccolithophore bloom in the northern North Sea, the DMS flux was equivalent to 1.3 % of gross DMSP production and 10 % of the DMS production in surface layers (Archer et al., 2002). Occasions when the sea-air flux might be quantitatively more important are during turbulent conditions (Simo and Pedros-Alio, 1999) and also in the upper metre of the water column.

The proportion of DMS that is lost to biological consumption, ventilation and photolysis varies over relatively short time-periods. One of the earliest studies on DMS consumption by Kiene and Bates (1990) reported that microbial utilisation exceeded ventilation. In 1996, Kieber et al. reported that UV photo-oxidation in the equatorial Pacific could occur at rates approaching microbial utilisation in the surface waters. Simó and Pedrós-Alió (1999) demonstrated that photolysis and ventilation of DMS were driven by radiation and wind-speed and these variables were subject to short-term physical variability.

#### 1.1.4 Marine emissions of DMS

The oceans are a source of aerosols which influence atmospheric radiative forcing due to their ability to scatter and absorb infrared radiation in the atmosphere and alter the properties of clouds. Many aerosol species *e.g.* sulphates and secondary organics, are not directly emitted from the sea surface, but form in the atmosphere from gaseous precursors, such as DMS and other biogenic hydrocarbons (IPCC, 2001). The upper water column is supersaturated when compared to atmospheric DMS and supports a sea-air flux of 15–33 Tg S yr<sup>-1</sup> (Kettle and Andreae, 2000). This represents >90 % of the oceanic non-sea salt sulphur flux and > 50 % of the global non-anthropogenic sulphur flux (Bates et al., 1992; Chin and Jacob, 1996).

In the atmosphere, DMS is rapidly oxidised by OH, NO<sub>3</sub><sup>-</sup> or IO radicals to produce methanesulphonic acid, sulphur dioxide and SO<sub>4</sub><sup>2-</sup> (Andreae, 1990; Barnes et al., 1991; Tyndall and Ravishankara, 1991). The oxidation products of DMS are involved in direct radiative forcing, as well as indirect forcing (Charlson and Wigley, 1994). Direct radiative forcing occurs when the small sub-micron sized particles scatter and absorb solar infrared radiation. These particles may serve as condensation nuclei for water vapour resulting in cloud formation. Indirect forcing occurs due to the alteration of cloud properties *e.g.* density and size, which consequently affects their reflectivity (Quinn et al., 1993).

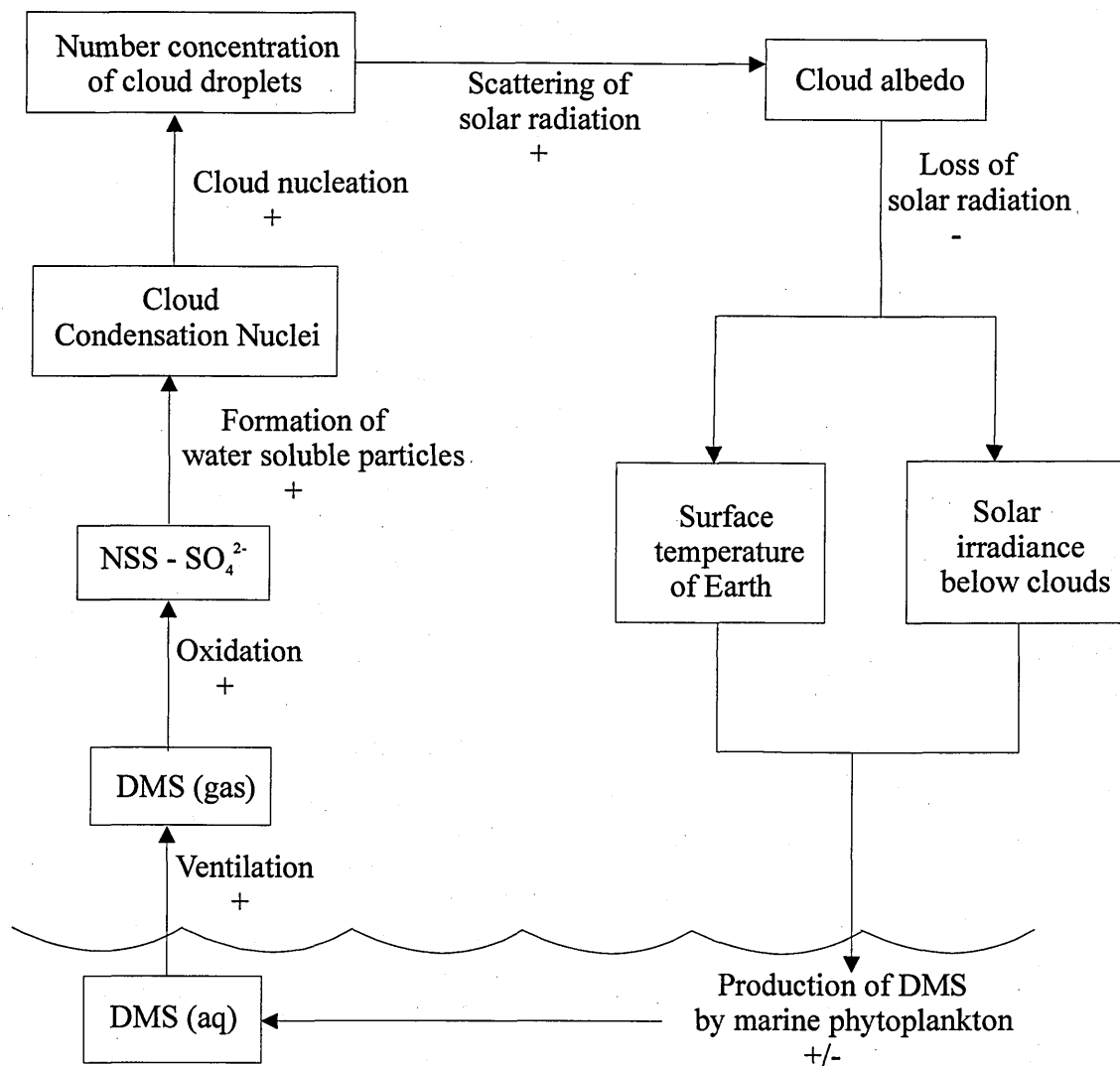
Aerosols are considered to have made a significant negative contribution to the overall radiative forcing (IPCC, 2001) and as such have a role in cooling the Earth's climate. However due to their short atmospheric lifetimes aerosols do not represent a long-term offset to the warming influences of greenhouse gases (Andreae, 1986; Chatfield and Crutzen, 1990; Gabric et al., 1996).

### 1.1.5 The CLAW hypothesis

The phytoplankton production of DMS and the subsequent sea-air flux was used to formulate the 'CLAW hypothesis' (named after the authors initials) (Charlson et al., 1987). In their original paper, Charlson, Lovelock, Andrae and Warren formalised the link between DMS and cloud albedo, and then proceeded to speculate that the system might display feedback control (Fig. 1.6). Therefore any change in climate might be counter-acted by a corresponding change in the DMS-Cloud Condensation Nuclei-Albedo effect. The original authors noted that the uncertain link in the proposed feedback loop is the effect of cloud albedo on DMS emissions.

The capability for a feedback loops between marine biota and climate mediated by DMS has provoked widespread scientific interest. Biogeochemical feedbacks which may reduce or amplify the net impact of the anthropogenic forcing are extremely important to the understanding and prediction of climate change (Lovelock et al., 1972). Both DMSP and DMS have been applied in large-scale modelling efforts to assess climate change scenarios (Bopp et al., 2003; Gabric et al., 2003). However although upper-ocean dynamics have been the subject of extensive research (Section 1.1) there are still large uncertainties both on the characteristics (+ or -) and on the magnitude of the feedback (Liss et al., 1994). A correlation between DMS and mixed-layer depths has been used as support of the feedback hypothesis, whereby a localised increase in solar radiation would stratify and shoal the surface mixing layer (Simó and Pedros-Alio, 1999). This would cause a stimulation of DMS production from the DMSP stock in the plankton assemblage as required for the feedback hypothesis. More recently Vallina and Simo (2007) observed a correlation between solar radiation and DMS concentrations from the analysis of a global database on

DMS surface seawater concentrations (<http://saga.pmel.noaa.gov/dms>). The authors concluded that the positive correlation further supports the negative feedback hypothesis as DMS emissions are driven by the light dose received by the pelagic ecosystem. The link between marine biota, DMS and climate has also been reviewed using results from an ice-core taken at the Vostok station in Antarctica. During the last glacial period, both methane sulphonic acid and non-sea salt sulphate were higher than in the previous interglacial (Legrand et al., 1991). Watson and Liss (1998) suggest that the marine biological changes which occurred over the last glacial-interglacial period were positive feedbacks. This suggests that changes in the climate system were reinforced by changes in the marine biota.



**Figure 1.6** Diagram of the CLAW hypothesis. From Charlson et al (1987).

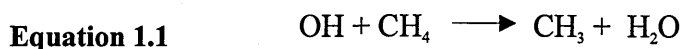
## **1.2 Methane**

In addition to the release of aerosols and their precursors from the marine environment, as discussed in previous section, the oceans are also a net source of methane. This section describes the role of methane in the Earth's atmosphere, and then considers biogenic methanogenesis within the wider context of anaerobic metabolism, before discussing the microbes responsible for producing methane as a metabolic by-product and the oxidative process.

### **1.2.1 Methane in the atmosphere**

In the atmosphere, methane acts as a greenhouse gas and influences tropospheric and stratospheric chemical cycling. The trapping of incoming solar radiation by non-conservative, radiatively active gases and particles is vital to life on Earth (Houghton et al., 1992). Solar radiation enters the Earth's atmosphere as short-wave ultraviolet radiation, of which approximately one-third is reflected back into space, whilst two-thirds are absorbed by the atmosphere and the Earth's surface (Ramanathan, 1988; Houghton et al., 1992). For the Earth to be in thermal equilibrium, the absorbed solar energy must be balanced by thermal energy loss. The most efficient process to lose energy from the Earth is through long-wave infrared radiation, between  $\lambda=8$  and  $12\text{ }\mu\text{m}$ , known as the atmospheric window (Kiehl and Trenberth, 1997). Because the longwave absorption of methane occurs within the atmospheric window,  $\lambda=7.6\text{ }\mu\text{m}$ , it is an effective greenhouse gas (Herzberg, 1945). Therefore although the atmospheric abundance of methane is less than  $\text{CO}_2$ , it has a relatively high warming efficiency, as quantified by its 'radiative forcing' (discussed further in Section 9.3).

In addition to its direct effect on radiative forcing, methane also plays an important role in atmospheric chemistry. The chemical removal of atmospheric methane in the troposphere is largely initiated by the reaction with hydroxyl radicals (OH) (Eq 1.1) (Fung et al., 1991; Lelieveld et al., 1998). This reaction accounts for the removal of approximately 75 % of the annual flux of methane to the troposphere (IPCC, 2001).

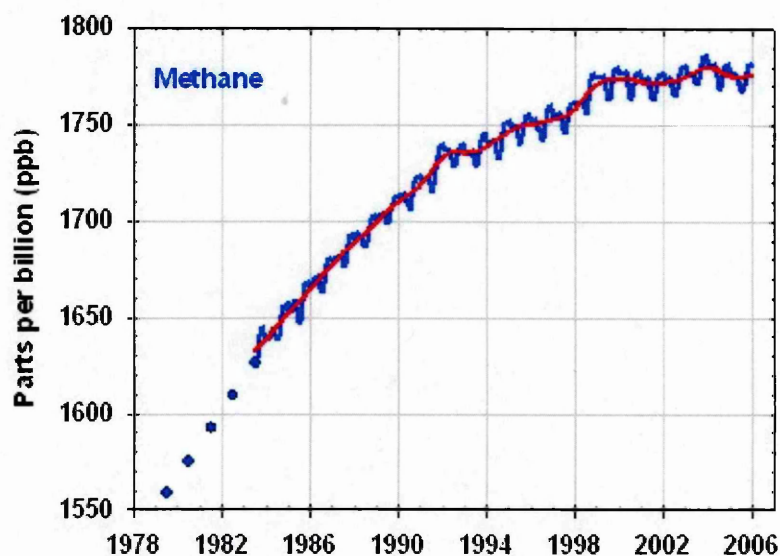


Increases of methane tend to decrease OH radicals, and since reduced [OH] increase the lifetime of methane, it produces a negative feedback (Johnson et al., 2001). Furthermore, because OH oxidation is a competitive process, the oxidation of methane influences the concentration of other gases such as O<sub>3</sub>, CO and H<sub>2</sub>O (Cicerone and Oremland, 1988). In the stratosphere, methane oxidation is an important source of stratospheric water vapour. Methane also reacts with Cl in the stratosphere resulting in the production of HCl, which is then available for the production of Cl and ClO (Cicerone and Oremland, 1988), which are both ozone-destroying compounds. Stratospheric loss of methane has been reported to account for approximately 40 Tg CH<sub>4</sub> yr<sup>-1</sup>, compared with an estimated loss of 506 Tg CH<sub>4</sub> yr<sup>-1</sup> via tropospheric OH (IPCC, 2001).

Atmospheric concentrations of methane have increased by a factor of ~2.5 since the pre-industrial era (Etheridge et al., 1998) (Fig. 1.7). Ice-core data show that the atmospheric mixing ratios of methane 200 years ago were approximately 700 ppbv increasing to current estimates of 1812 ppbv. However, despite rising anthropogenic input of methane, the increase rate in atmospheric methane concentrations is declining (Fig. 1.7). There is no clear quantitative explanation for the observed trend, which is summarised in the IPCC



(2001) report and has been attributed to decreases of UV in the tropics, decreases in wetland methane emissions or due to increases in tropospheric OH radical due to ozone depletion. Most recent reports conclude that based on current knowledge of the global methane budget and its changes, it is not possible to tell if the atmospheric methane burden has peaked, or if we are only observing a persistent, but temporary pause in its increase (Dlugokencky et al., 2003).



**Figure 1.7** Global averages of methane concentrations from the NOAA global flask sampling network since 1978. Taken from: <http://www.esrl.noaa.gov/gmd/aggi>.

### 1.2.1 Overview of anaerobic metabolism

Although atmospheric methane concentrations have increased with anthropogenic activity, it is thought that the Archean Earth (2.5 billion years ago) had elevated atmospheric concentrations of methane contributing to a possible global warming of the Earth's surface (Pavlov et al., 2000). Some of the earliest life forms on the Earth were Archaea; micro-organisms capable of existence in the absence of oxygen by decomposing organic matter and converting the carbon to methane. After the evolution of oxygenic photosynthesis the

Earth's atmosphere and the oceans became oxygenated and anaerobic metabolism was gradually supplanted by aerobic metabolism (Catling et al., 2001). Despite the transformation to a predominantly oxidised planet, anaerobic environments have persisted on the Earth because of the combined influence of organic matter and water. Molecular oxygen diffuses about  $10^4$  times more slowly through water than air, and organic matter supports a large biotic demand that consumes supply faster than is replaced by diffusion (Migonigal et al., 2004).

One of the most fundamental differences between aerobic and anaerobic metabolism is energy yield (Table 1.1). In contrast to aerobic respiration, due to the low energy yield associated with anaerobic metabolism, no single anaerobic micro-organisms can completely degrade organic polymers to  $\text{CO}_2$  and  $\text{H}_2\text{O}$ . Therefore under anaerobic conditions, the mineralization of organic carbon to  $\text{CO}_2$  is a multi-step process that involves a consortium of organisms (Fig. 1.8).

**Table 1.1** Comparison of the energetic yield from aerobic and anaerobic metabolism which refer to the oxidation of glucose under aerobic conditions<sup>1</sup>, compared to typical methanogenic conditions<sup>2</sup>. From Migonigal et al. (2004).

Metabolism	Reactants		Products	$\Delta G$ (kJ mol <sup>-1</sup> )
Aerobic	$\text{C}_6\text{H}_{12}\text{O}_6 + 6\text{O}_2$	→	$6\text{CO}_2 + 6\text{H}_2\text{O}$	-2,900 <sup>1</sup>
Anaerobic	$\text{C}_6\text{H}_{12}\text{O}_6$	→	$3\text{CO}_2 + 4\text{CH}_4$	-400 <sup>2</sup>

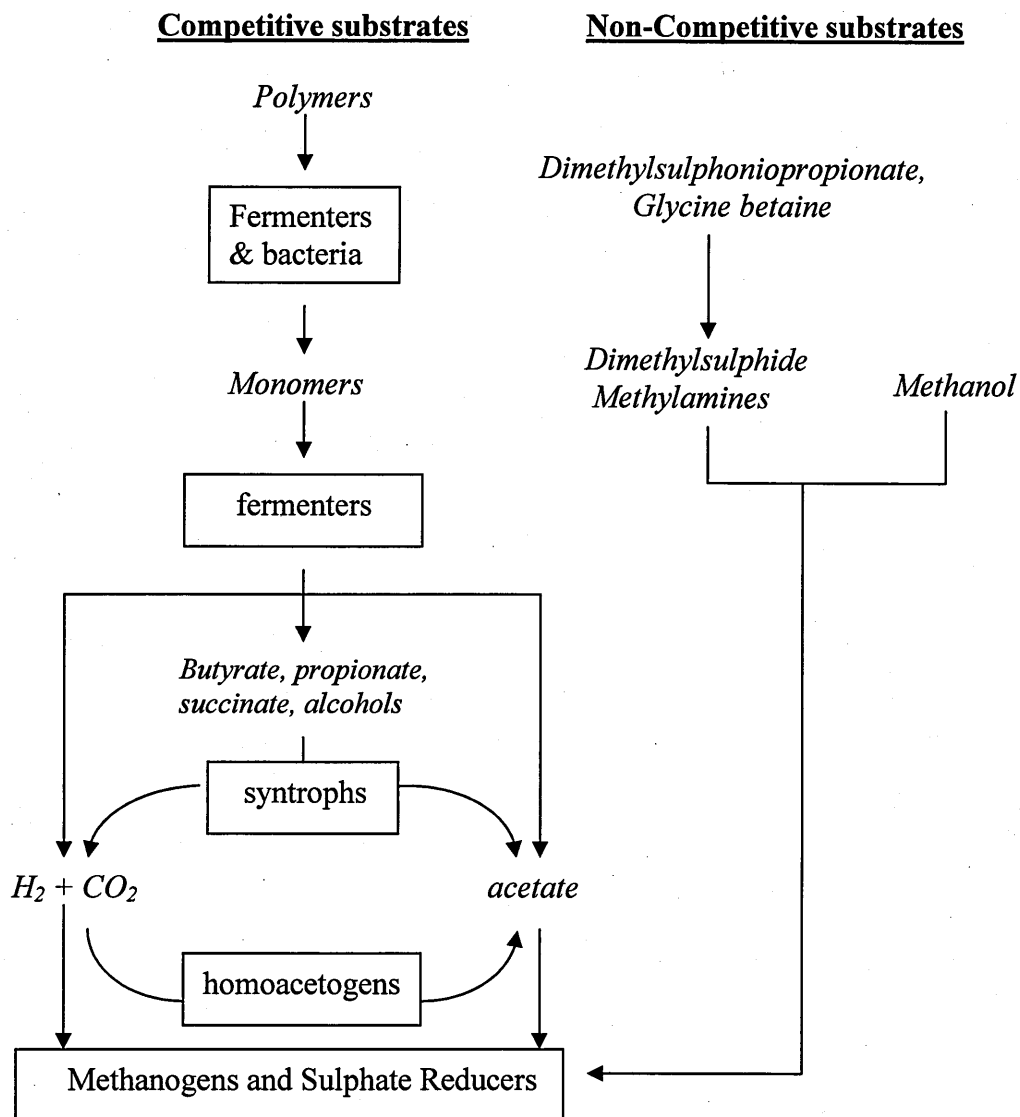
The first step in anaerobic decomposition is the breakdown of high molecular weight organic molecules *e.g.* proteins and carbohydrates, into monomers. Depending upon the

quality of organic matter, this initial polymer degradation can be rate limiting to overall anaerobic decomposition (Wu et al., 2001; Glissmann and Conrad, 2002), although this is not as common in marine ecosystems as algal cells are largely composed of carbohydrates and therefore more labile (Benner et al., 1992).

The next step is fermentation where organic compounds serve as both the electron acceptor and donor. Primary fermentation is the exergonic breakdown of monomers *e.g.* amino acids, glucose to products such as alcohols, fatty acids, hydrogen and CO<sub>2</sub>. These fermentation products can then be utilised directly by other anaerobes, or alternatively secondary fermentation can further convert these primary products to acetate and low molecular-weight products. The fermentative process is a key component of the anaerobic mineralization process as non-fermenting anaerobes can not utilise the monomers released during the initial polymer hydrolysis (Magonigal et al., 2004). Molecular hydrogen (H<sub>2</sub>) is the most abundant product of the fermentation process and is the most common electron donor in terminal anaerobic metabolism (Conrad, 1999). However, the secondary fermentation process can be limited if H<sub>2</sub> accumulates in the system and the reaction becomes endergonic. This rarely occurs because H<sub>2</sub> is removed by H<sub>2</sub>-consuming micro-organisms *e.g.* methanogens and sulphate-reducers (Zinder and Koch, 1984; Conrad et al., 1986). Therefore, two metabolically different types of microbes can depend upon each other for the degradation of a substrate (Biebl and Pfennig, 1978) in a process referred to as 'interspecies hydrogen transfer' when H<sub>2</sub> is involved (Thiele and Zeikus, 1998).

Anaerobic micro-organisms compete for common electron donors, such as acetate and H<sub>2</sub>. Sulphate-reducing bacteria typically inhibit H<sub>2</sub>-dependent methanogens, by reducing concentrations to a value below the threshold at which CH<sub>4</sub> production is

thermodynamically feasible (Lovley et al., 1982). Therefore in anaerobic sulphate-abundant environments, sulphate reduction dominates terminal carbon metabolism (Capone et al., 1983). However there are instances when methanogenesis and sulphate-reduction are not mutually exclusive and can occur simultaneously. One such example appears to be when methanogens metabolise methylated compounds (Section 1.2.2) as sulphate-reducing bacteria show little apparent affinity for methanol or methylamines. The occurrence of methylotrophic methanogenesis in the presence of sulphate-reduction has led to the descriptive labelling of methylated molecules as 'non-competitive compounds' (Oremland et al., 1982; King, 1984a, b) (Fig. 1.8).



**Figure 1.8** Diagram of anaerobic decomposition of organic matter. Modified from Conrad (1999)

### 1.2.2 Methanogenesis

Methanogenesis is the final step in the anaerobic pathway. In contrast to their phylogenetic diversity, methanogens use a relatively limited variety of substrates for metabolism including  $H_2$ , acetate, formate, primary and secondary alcohols and methylated compounds (Zinder, 1993). Based on substrate use, methanogens are frequently classified into three functional groups: hydrogenotrophs; acetotrophs; and methylotrophs (Table 1.2).

Hydrogenotrophy is most common among the methanogens, whilst acetotrophy dominates in the natural environment. To date, only the Methanosarcinaceae family have been identified as exhibiting all three metabolic pathways and other methanogens are limited to one metabolic pathway (Mukhopadhyay, et al., 1993). All three metabolic pathways converge on the reduction of methyl-coenzyme M to methane (Fig. 1.9) which is catalysed by the enzyme methyl-coenzyme M reductase (Thauer, 1998). The *mcrA* gene which encodes for this enzyme is used as a specific functional marker gene in molecular ecological analyses (Leuders et al., 2001; Hallam et al., 2003).

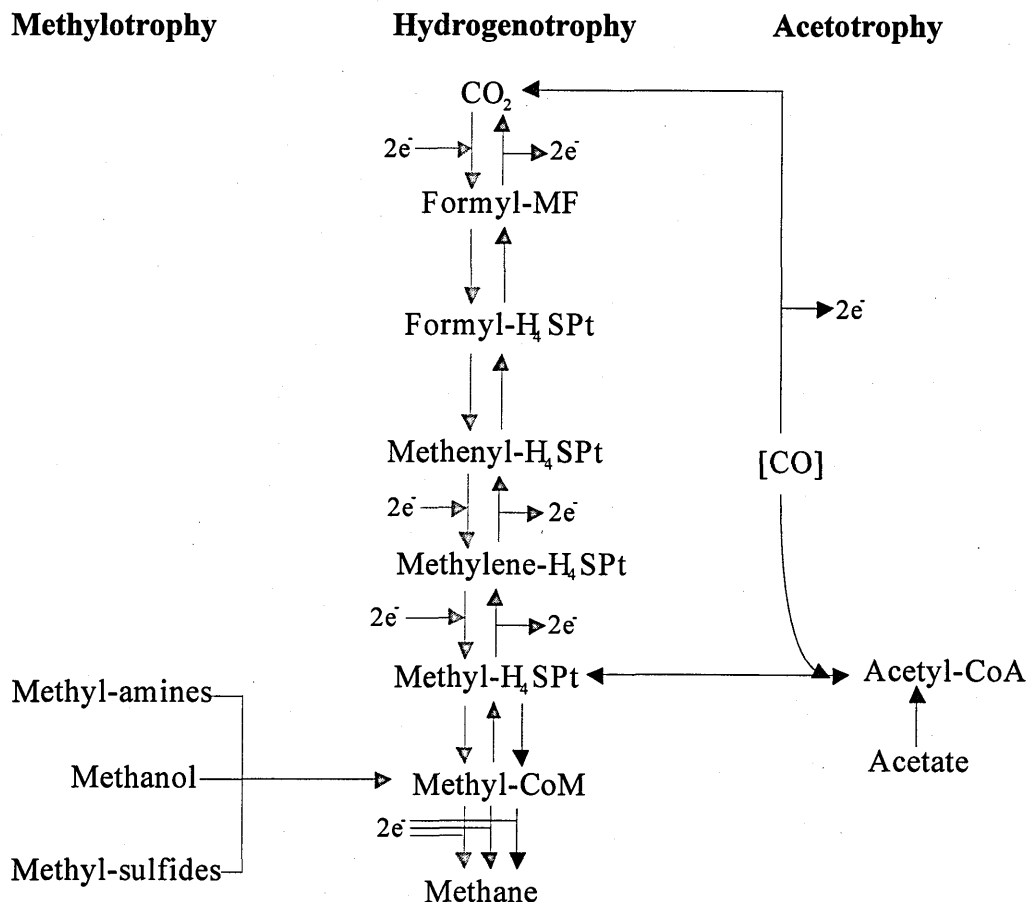
**Table 1.2** Different methane-producing metabolic pathways. From Fenchel and Finlay (1995)

Metabolism	Reactants		Products	$\Delta G$ (kJ mol <sup>-1</sup> )
Hydrogenotrophy	CO <sub>2</sub> + 4H <sub>2</sub>	→	CH <sub>4</sub> + 2H <sub>2</sub> O	-136
Acetotrophy	CH <sub>3</sub> COOH	→	CH <sub>4</sub> + CO <sub>2</sub>	-31
Methyлотrophy	4CH <sub>3</sub> OH	→	3CH <sub>4</sub> + CO <sub>2</sub> + 2H <sub>2</sub> O	-105

Hydrogenotrophy (or CO<sub>2</sub> reduction) is widespread among the methanogens, whereby H<sub>2</sub> is the source of both energy and electrons, and CO<sub>2</sub> is both an electron sink and the source of cellular carbon (Garcia et al., 2000). CO<sub>2</sub>/H<sub>2</sub> reduction requires a 4:1 molar ratio of H<sub>2</sub> to CO<sub>2</sub>, however in the natural environment H<sub>2</sub> is typically at nanomolar concentrations, while CO<sub>2</sub> is at millimolar concentrations. Therefore, substrate limitation of methane production is due to the lack of H<sub>2</sub> as the electron donor (Megonigal et al., 2004).

Approximately 45 % of hydrogenotrophic methanogens can substitute formate for H<sub>2</sub> (Thiele and Zeikus, 1998; Garcia et al., 2000). The co-enzyme methanofuran is involved in the initial step of CO<sub>2</sub>/H<sub>2</sub> reduction to form a formyl group which is sequentially

reduced through methenyl and methylene to methyl (Fig. 1.9). Methyl is subsequently transferred to coenzyme M and finally reduced to free methane (Thauer, 1998).



**Figure 1.9** The three metabolic pathways for methanogenesis found in methanogens. From Galagan (2002)

Acetotrophy (or acetoclastic, acetate fermentation) is restricted to two genera of methanogens: *Methanosarcina* and *Methanosaeta*, which comprise ~10 % of methanogen species (Migonigal et al., 2004). Despite its limited taxonomic distribution, approximately two-thirds of global methane production is thought to result from acetotrophy and it is the dominant methanogenic pathway in many freshwater environments (Whiticar et al., 1986; Conrad, 1999; Le Mer and Roger, 2001; Brooks Avery Jr et al., 2003). A prominent feature of acetoclastic methanogenesis is the low isotopic fractionation of carbon between

20-30 %, compared to methane production via CO<sub>2</sub> reduction of 55-60 % (Krzycki et al., 1987; Gelwicks et al., 1989). Acetotrophy can be considered a specialized form of methylotrophy, in which the molecule is cleaved and the methyl group is reduced to methane with electrons derived from the oxidation of the carboxyl group to CO<sub>2</sub> (Pine and Barker, 1956; Fenchel and Finlay, 1995; Hornibrook et al., 2000).

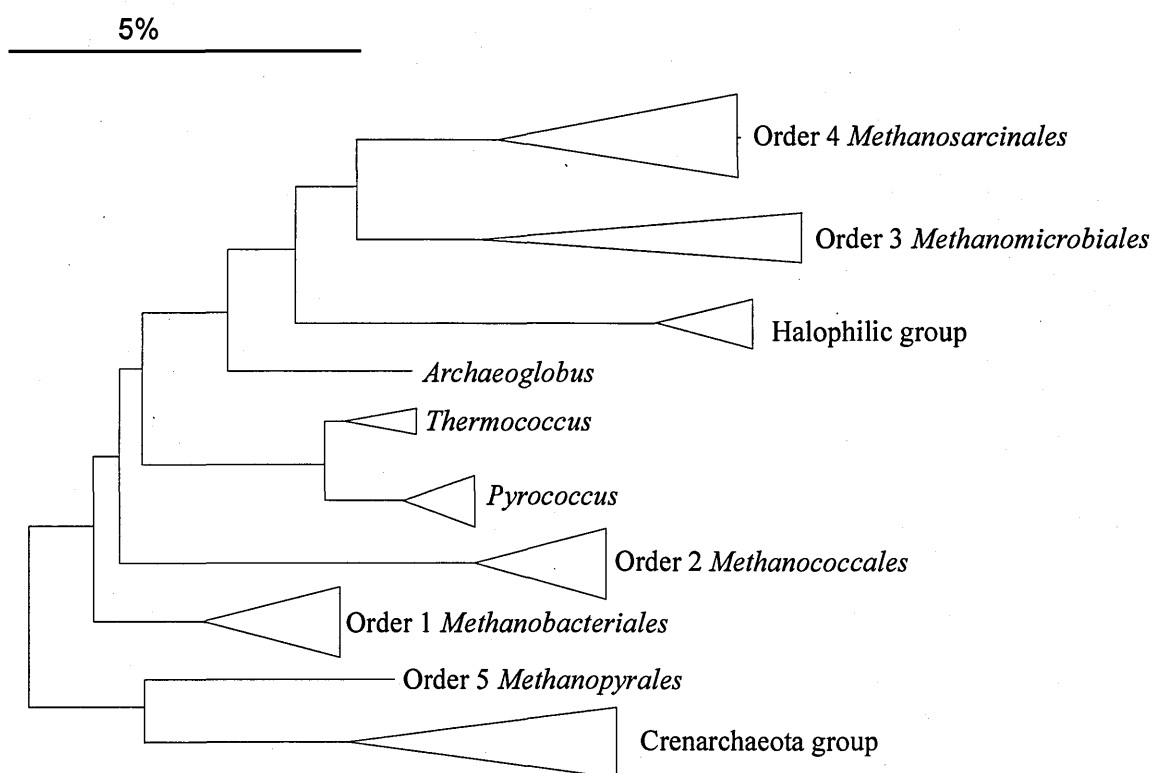
About 26 % of methanogen species can use methylated substrates other than acetate, such as methanol, methionine, methylated amines and methylated sulphur compounds (dimethylsulphide, dimethyldisulphide, methanethiol) (Hippe et al., 1979). In depth reviews on methylotrophy are provided by Kiene (1991) and Keltjens and Vogels (1993), and is discussed further in Chapter 6.. In brief, methylotrophy is a type of fermentation as it does not require an external electron acceptor. Methanogens that utilize methyl compounds transfer the methyl group to CoM for reduction to methane; and concomitant oxidation of one molecule of the C<sub>1</sub> compound to CO<sub>2</sub> (Fig 1.9). The oxidation pathway generally proceeds by the same sequence of reactions as the hydrogenotrophic pathway, albeit in reverse order and with slightly modified one-carbon and electron carriers. The oxidation pathway provides the cell with methyl-H<sub>4</sub>SPt and CO<sub>2</sub> required for the synthesis of acetyl-CoA and reducing equivalents are generated for anabolic reduction reactions and the reduction of CoM-S-S-HTP.

### **1.2.3 Methanogen phylogeny, characteristics and habitats**

Only a few micro-organisms, referred to as ‘methanogens’, produce methane as a by-product of their metabolism. Methanogens are currently the largest collective group within the Archaea kingdom with unique biochemical characterisations, distinct from Bacteria and Eucarya (Woese et al., 1990). For example, the cell walls of methanogens do not



contain the peptidoglycans characteristic of other micro-organisms, but instead consist of group-specific lipids such as isoprenoids or squalenes depending on the methanogen species (Langworthy, 1985). In addition, a number of unique coenzymes have been discovered in methanogens including methanofuran, deazaflavin F<sub>420</sub>, Coenzyme M and HS-coenzyme (Rouviere and Wolfe, 1988). Based on 16S rRNA gene analysis and supported by biochemical evidence, five orders of methanogens have been identified and characterised (Fig. 1.10) as described in Bergey's Manual of Systematic Bacteriology (2001) and discussed in Boone et al. (1993), Garcia et al. (2000)



**Figure 1.10** Phylogeny of the methanogens, kingdom Archaea, highlighting the five methanogen orders. The triangles represent different clades within the Archaea and the size of individual triangles is proportional to the number of sequences within the clade. From Garcia (2000).

- Order Methanobacteriales contains two families: Family *Methanobacteriaceae* has four morphologically distinct genera and use  $H_2/CO_2$ , 2-propanol, formate and methanol as precursors for methanogenesis. Family *Methanothermaceae* consists of a single genus of extreme thermophile hydrogenotrophs.
- Order Methanococcales contains two families *Methanococcaceae* and *Methanocaldococcaceae* mainly from marine and coastal environments. Most of the species have been identified as able to use both  $H_2$  and formate as electron donors
- Order Methanomicrobiales contains three families (*Methanomicrobiaceae*, *Methanocorpusculaceae*, *Methanospirillaceae*) and nine genera of hydrogenotrophic methanogens.
- Order Methanosarcinales consists of all the acetotrophic and/or methylotrophic methanogens, grouped into two families. Family *Methanosarcinaceae* consists six genera of the versatile methanogens able to use  $H_2/CO_2$ , acetate or methyl compounds as substrate, although only the genus *Methanosarcina* demonstrate the potential of all three metabolic pathways. Family *Methanosaetaceae* includes one genus of obligate acetotrophic methanogens
- Order Methanopyrales consists of a single family and species of hyperthermophilic methanogens which are unrelated to all other methanogens.

Methanogens require a low reducing potential (Eh below -0.33V) and are reputed as being strict anaerobes (Hungate, 1967). Most strains of methanogens are thought to die even when exposed to low concentrations of oxygen due to an irreversible disassociation of their enzyme complex (Jones et al., 1987). However there is evidence that certain strains can maintain viability for up to 24 hours when exposed to air (Kiener and Leisinger, 1983). Furthermore, biochemical analysis has revealed the presence of various anti-oxidant

enzymes *e.g.* catalases and super-oxide dismutases, in a select number of methanogens including *Methanobacterium bryantii* (Kirby et al., 1981). These antioxidants provide defence against the toxic effects of products resulting from the incomplete reduction of oxygen.

The temperature range for methanogenesis is broad, ranging from 4-110 °C. Most methanogens are mesophilic with a temperature optima of ~35 °C, although thermophily is relatively common in methanogens which is not unusual considering it is widespread in the Archaea (Woese, 1987). Almost 25 % of methanogens are classified as thermophilic including *Methanopyrus kandleri* (Kurr et al., 1991) which was isolated from shallow marine hydrothermal system and has an optimum temperature near 100 °C. Methanogens grow over a relatively narrow pH range (6 to 8) (Jones et al., 1987) although *in situ* methanogenesis has been demonstrated in acid environments such as peat bogs (Williams and Crawford, 1985). Methanogens can be found in freshwater to hypersaline environments. The known halophilic methanogens belong to the Methanosarcinaceae *e.g.* *Methanohalophilus* (Mathrani et al., 1988). Like other micro-organisms, methanogens adapt to salinity by increasing compatible solutes *e.g.* glycine betaine, in their cytoplasm to equalise the internal and external osmolarity (Zinder, 1993)

The methanogens are widely distributed in nature, but are typically considered to be confined to strictly anaerobic environments. The three main natural methanogen ecosystems, as described by Garcia et al. (2000), include.

- Soils and aquatic environments. Methanogen populations have been characterised in a wide variety of freshwater (Leuders et al., 2001; Galand et al., 2003) and marine sediments (Purdy et al., 2002; Castro et al., 2004). In freshwater environments such as

paddy fields and lake sediments, acetotrophy can be the dominant methanogenic pathway.

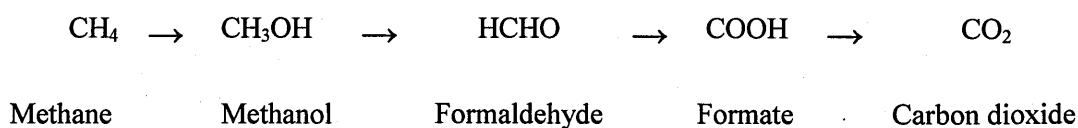
- Within living organisms. Methanogens occur within the digestive tracts of a variety of animals including herbivorous mammals, insects, arthropods (as reviewed by Hackstein (1997)) and can be directly involved in the digestive processes. In particular, the hindguts of termites are a significant source of methane to the atmosphere (IPCC, 2001).
- Extreme environments. Thermal environments such as hot springs or hydrothermal vents are sites of active methanogenesis and methanogens have recently been isolated from submarine vent systems (Jeanthon et al., 1999; Takai et al., 2002). Biogenic methane has also been detected in hypersaline environments (Rengpipat et al., 1988) and also oil-well waters (Belyaev et al., 1983).

#### **1.2.4 Methane oxidation.**

An understanding of methanogenesis is not complete without considering the oxidation process. The oxidation of methane by micro-organisms is a very important process in the global methane budget. Methane produced in anaerobic environments will escape to the atmosphere if it is not oxidised first by methanotrophs. Estimates suggest that methanotrophy consumes approximately 80 % of the gross methane produced (Reeburgh, 1996) highlighting the important role of these microbes. Methane oxidation can occur both aerobically and anaerobically.

Aerobic methane-oxidising bacteria (methanotrophs) are a diverse group of bacteria that are currently represented by 13 recognised genera that belong to the  $\alpha$  and  $\gamma$  subdivisions

of the *Proteobacteria*. A full review of methanotrophy including their taxonomy, physiology and ecology is provided by Hanson and Hanson (1996). In brief, methanotrophs are a subset of methylotrophic bacteria, and restricted to metabolising  $C_1$  compounds. They are unique in their ability to use methane as a sole carbon and energy source. Methane is oxidised in a multi-step process to eventually yield carbon dioxide (Fig. 1.11). The first stage involves the enzyme methane monooxygenase to produce methanol from methane and oxygen. The oxidation then proceeds through the production of formaldehyde using methanol dehydrogenase. The subsequent oxidation of formaldehyde to formate and then  $CO_2$  is also catalysed by dehydrogenase enzymes. Aerobic methanotrophs occur at the oxic-anoxic interface of methane-producing habitats e.g. sediments (King, 1990), animals (Jahnke et al., 1998), inside wetland plants (Bosse and Frenzel, 1997) and aquatic water-columns (Sieburth and Donaghay, 1993).



**Figure 1.11** Oxidation pathway of methane

In addition to aerobic oxidation of methane, the oxidative process is also known to occur anaerobically. The anaerobic oxidation of methane was first proposed in the mid-1970's when it was established that in certain horizons in sediments and waters at continental margins the net consumption of methane occurs (Martens and Berner, 1974; Barnes and Goldberg, 1976; Reeburgh, 1976). The correlation in depth at which anaerobic oxidation of methane occurred with increased sulphate reduction suggested sulphate was the terminal electron acceptor for this process (Barnes and Goldberg, 1976) (Eq. 1.2):



In contrast with aerobic methane oxidation, the exact processes of the anaerobic counterpart are still being resolved. Biogeochemical studies have shown that the overall process involves a transfer of electrons from methane to sulphate (Iversen and Jørgensen, 1985; Hoehler et al., 1994). The isotopic and genetic signature of the microbial biomass in these environments shows that this transfer is probably mediated by a microbial consortium that includes two major groups of methanogen-related Archaea that co-occur with sulphate-reducing bacteria (Hinrichs et al., 1999; Boetius et al., 2000). The microbes identified to date belong to the order Methanosarcinales (ANME-2 group), and the *Desulfosarcina/Desulfococcus* branch of the  $\delta$ -proteobacteria (Boetius et al. 2000a; Orphan et al. 2001b). A second archaeal group (ANME-1), distantly related to Methanosarcinales, have also been shown to mediate the anaerobic oxidation of methane (Michaelis et al. 2002; Orphan et al. 2002).

### 1.2.5 Methane in the global ocean

Methane concentrations in the upper water column are determined by a combination of externally derived sources, oxidative processes, the sea-air flux, and *in situ* production. The allochthonous inputs include: the production of methane in marine sediments during the diagenesis of organic carbon; hydrothermal vent systems; methane clathrate hydrate decomposition; marine seeps, vents and mud volcanoes; and transport from methane-rich areas *e.g.* coastal regions (Dickens, 2003; Kennett et al., 2003; Kvenvolden and Rogers, 2005; Reeburgh, 2007). There is a growing recognition that methane represents a large

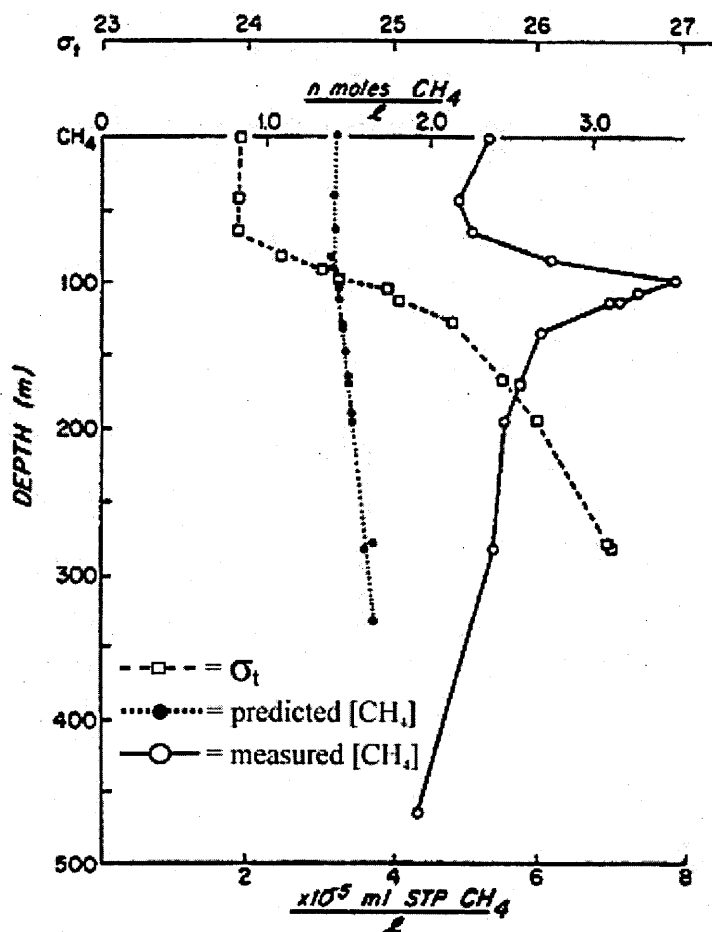
carbon reservoir within the sea-floor. The abundance of carbon residing in gas hydrates and free gas has been estimated at 200-10,000 Gt (Kvenvolden and Lorenson, 2001; Buffet and Archer, 2004; Milkov, 2004). It is also emerging that the sedimentary source of methane is a dynamic reservoir, which can be released into the ocean at high (millimolar) concentrations. For example at the Håkon Mosby Mud Volcano, a methane flux of 0.08-0.36 mol CH<sub>4</sub> sec<sup>-1</sup> was estimated by Sauter et al. (2006). In addition the dissolution of methane from hydrate has been estimated at  $0.37 \pm 0.03$  mmol CH<sub>4</sub> m<sup>-2</sup> sec<sup>-1</sup> (Rehder et al., 2004). Following the release of methane to the overlying water column, the oceans may act as a sink (discussed below) or as a conduit. If methane released from the sea-floor does pass through the water column into the atmosphere, it acts as a greenhouse gas (Section 1.2.1). The dissociation of marine sedimentary methane clathrate hydrates have been implicated in episodes of rapid climate change including the Paleocene thermal maximum (Dickens et al., 1997) and the climate oscillations in the late Quaternary (Kennett et al., 2003).

Once methane is released into the oceans the amount of methane that is available for air-sea exchange depends upon the water column oxidation rates. There have been relatively few measurements on aerobic water column oxidation rates compared to their anaerobic counterpart. The first estimates of methane consumption in the ocean were made by Scranton and Brewer (1977) who related oxidation rates to the age of different water masses. The authors found that oxidation rates in the first decade were 0.15 nmol CH<sub>4</sub> yr<sup>-1</sup> and subsequently decreased to 10<sup>-4</sup> nmol yr<sup>-1</sup> for seawater older than 150 years. However these measurements were taken in seawater with ambient methane concentrations of 1.2-2.3 nmol dm<sup>-3</sup> CH<sub>4</sub> (Scranton and Brewer, 1978). At marine locations where elevated CH<sub>4</sub> concentrations are observed, much higher oxidation rates have been observed. For

example, the oxidation of methane at a deep-sea hydrothermal plume in the Atlantic was  $0.15 \text{ nmol CH}_4 \text{ d}^{-1}$ , corresponding to a turnover time of 1 week (de Angelis et al., 1993). In addition, the oxidation rates of methane emitted from decomposing methane hydrates in Eel River Basin, northern California were calculated at a turnover rate of 1.5 yr compared to a turnover rate of decades in the upper part of the water column (Valentine et al., 2001).

Within the marine environment, one of the most enigmatic processes regarding methane biochemical cycling is the supersaturation in the surface mixed layer. Methane concentrations of  $\sim 5 \text{ nmol}$  supersaturated with respect to the atmosphere have been observed in many parts of the world's oceans (Fig. 1.12) (Scranton and Brewer, 1977; Traganza et al., 1979; Burke et al., 1983; Ward et al., 1987). Furthermore, the sea-air flux of methane typically exceeds the rate of methane migration across the thermocline into the mixed layer (Scranton and Brewer, 1977; Tilbrook and Karl, 1995; Holmes et al., 2000; Oudot et al., 2002). Therefore an *in situ* methane production mechanism is required to explain the observed supersaturations.





**Figure 1.12** *In situ* methane production within the upper water column. Taken from Scranton and Brewer (1977)

Methane production in the open ocean is unexpected due to the presence of oxygen.

Methanogens are reputed to be fastidious anaerobes that die in the presence of oxygen (Hungate, 1967). In addition, sulphate is a major constituent of seawater (28 mmol) and data from anoxic marine sediments indicate that methanogenesis does not occur until sulphate is nearly exhausted and sulphate reduction rates decrease (Martens and Berner, 1977; Crill and Martens, 1983). This suggests that almost all of the 28 mmol ocean water column sulphate pool must be reduced before conditions favourable for methanogenesis are obtained. At the present time, there is no consensus explanation for methanogenesis in the upper ocean and a variety of explanations exist to resolve the paradox:

1. Methane could originate in the guts of zooplankton or higher trophic organism, *e.g.* fish (Oremland, 1979). More recently, the release of gas by Atlantic and Pacific herring has been reported, though the gas was not identified (Wilson et al., 2003), suggesting marine fish could be a source of methane similar to ruminants. Methane have been correlated with zooplankton in the field (Traganza et al., 1979) and also in the laboratory (de Angelis and Lee, 1994).

2. Methane production is associated with phytoplankton, either directly or indirectly. There are at least five reported occurrences of methane being detected in algal cultures including *Rhodomonas lens*, *Prorocentrum minimum* (de Angelis and Lee, 1994), *Thalassiosira pseudonana* and *Emiliana huxleyi* (Scranton and Brewer, 1977) and cyanobacteria (Marty et al., 1997). These observations of methane have been used contrastingly by the authors as evidence of anaerobic microniches associated with algae (Marty et al., 1997) and also algal synthesis of methane (Scranton and Brewer, 1977). One recent discovery is that terrestrial plants can produce methane under oxic conditions (Keppler et al., 2006). Although the exact mechanism for methane production is not known, emissions were related to the quantity of pectin within the plant. It is possible that a similar biochemical pathway for methane production under oxic conditions exists in marine algae.

3. The most prominent and frequently postulated hypothesis in explanation to the methane paradox is the presence of reducing micro-environments associated with particulate matter environment (Jørgensen, 1977; Alldredge and Cohen, 1987; Paerl and Prufert, 1987). In support of this hypothesis, methane production has also been measured in samples of

particulate material, copepods and algae collected from the pelagic environment and incubated anaerobically (Oremland, 1979; Bianchi et al., 1992; Marty, 1993; Marty et al., 1997). Methanogens have also been isolated from the particulate material collected from the pelagic environment (Cynar and Yayanos, 1991; Sieburth, 1993). In addition, during laboratory grazing experiments of copepod species grazing on phytoplankton, de Angelis and Lee (1994) detected the production of methane which they attributed to the presence of methanogens in the digestive tracts of zooplankton. In field-based measurements, methane was measured in sediment traps which collected sinking particulate material less than 200  $\mu\text{m}$  in diameter (Karl and Tilbrook, 1994).

In particular, Sieburth has supported the concept of methane production occurring within reduced microniches and detailed descriptions of the upper ocean methane cycle and the bacterial consortium that facilitate methanogenesis are described in Sieburth (1988), Sieburth (1991) and Sieburth (1993). One of the earlier reports by Sieburth (1983) suggested upper water column methane production could be considered as a 'false benthos', whereby bacterial processes in the pycnocline resemble those in sediments. Sieburth (1993) later reversed his hypothesis to propose that the benthos represents a more limited version of the pycnocline whereby anoxia in the sediments excludes the oxidative processes occurring and methane can therefore accumulate. The oxidation of methane makes the upper ocean methane cycle particularly difficult to elucidate. To date, there has been one published account of a methanotroph, *Methylophaga* sp. isolated from the upper water column (Sieburth et al., 1987)

It has been debated whether the methane produced in anoxic micro-niches is sufficient to account for the supersaturation in the surface mixed layer. Tilbrook and Karl (1994)

estimated the particle-to-seawater methane flux ( $\sim 40\text{--}1400 \text{ nmol m}^{-2} \text{ d}^{-1}$ ) was sufficient to produce the methane supersaturation observed in less than a month and replace the methane in the upper water column in 50 days. A similar conclusion was supported by the results of a recent vertical advection-diffusion model involving methane release from settling zooplankton faecal pellets (Nihous and Masutani, 2006). The authors of the model also noted the importance of including methane oxidation to account for the  $\delta^{13}\text{C}\text{-CH}_4$  values observed by Holmes et al. (2000). Contrastingly, Rudd and Taylor (1980) suggest that insufficient methane is produced by faecal pellets to account for the mixed layer supersaturation and the authors proceed to speculate on other potential sources of methane, as described previously. Rudd and Taylor (1980) also highlight that a major issue associated with methane production within anoxic microniches is the limitation of such small particles to facilitate oxygen depletion. Particles smaller than  $500 \mu\text{m}$  are considered unlikely to be anaerobic due to the size, respiration and organic matter content restraints (Ploug et al., 1997). This is discussed in much greater detail in Section 9.1.

If oxygen depletion does occur within particulate material, an additional explanation is required for the production of methane in the presence of sulphate. Laboratory experiments involving lake, estuarine and marine sediments have revealed how the addition of methanol, methionine, methylated amines, dimethylsulphide and methanethiol stimulated methane production (Oremland et al., 1982; Oremland and Polcin, 1982; King, 1984a; Kiene et al., 1986). In the marine environment, methylated amines and methylated sulphonium compounds can be produced via the decomposition of the algal compounds betaine and DMSP respectively (Section 1.1). In support of methylotrophic methanogenesis occurring in the upper water column, methanogens have been isolated from oxygenated seawater samples supplemented with mono-methylamines (Cynar and

Yayanos, 1991; Sieburth, 1993). However, so far there has been little consideration of the potential for methylotrophic methanogens in the upper water column to metabolise DMSP even though it is present at high concentrations (Section 1.1).

### 1.3 Outline of thesis

The aim of this thesis was to investigate if DMSP, DMSO and DMS could be metabolised by methanogenic Archaea within anaerobic microniches in the upper ocean. An experimental approach was adopted to investigate the sequences of events leading from the algal production of methylated sulphur, degradation by grazing and subsequent availability for methanogens, as portrayed in Figure 1.13. Specifically the thesis will:

- A. Quantify the production of DMSP and DMSO in cultures of marine phytoplankton belonging to major taxonomic groups (Chapter 3),
- B. Quantify the release of algal-DMSP and DMSO during grazing of phytoplankton by copepods and the concentration of DMSP and DMSO in faecal pellets (Chapter 4),
- C. Investigate the natural levels of DMSP and DMSO in particulate material in the water column (Chapter 5),
- D. Investigate whether DMSP, DMS and DMSO are metabolised by methanogens in particulate material collected from the pelagic environment (Chapter 6),
- E. Characterise the methanogenic community associated with zooplankton faecal material collected from copepods (Chapter 7),
- F. Investigate the association of methanogens with zooplankton (Chapter 8).

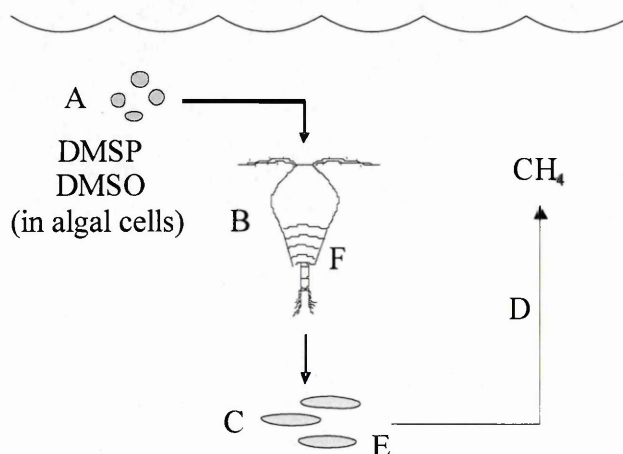


Figure 1.13 Diagram highlighting the investigative approach adopted by this thesis

## Chapter 2. Materials & Methods

### 2.1 Sulphur analysis.

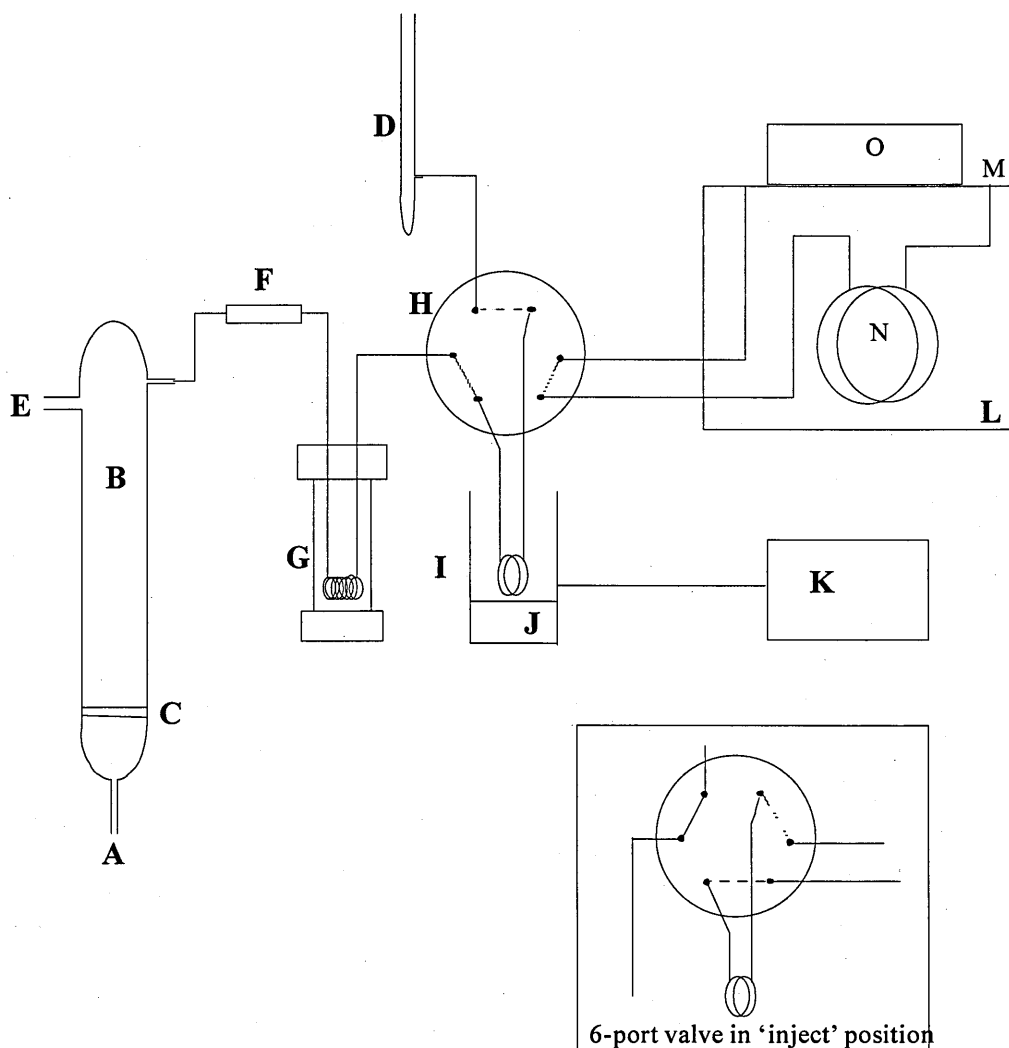
#### 2.1.1 Analytical equipment for sulphur analysis

The analytical procedure for sulphur compounds can be divided into two parts; the sample preparatory system and the sample analysis.

##### *Sample preparation system*

All components of the sample preparation system were connected using 1/8" or 1/16" o.d. PTFE tubing (Omnifit Ltd) (Fig. 2.1). Oxygen-free nitrogen (BOC; OFN grade) was used as the purge gas (A) and passed directly into a purge chamber (B) with a glass frit at the base (C). The flow rate ( $60 \text{ cm}^3 \text{ min}^{-1}$ ) was controlled using a needle valve and determined with a bubble flow-meter (D). The purge chamber removable top incorporated a sample inlet tube (E) and a purge gas outlet. Samples were loaded directly into the purge chamber via a two way valve, with luer connections. After purging, gas was directed through a glass tube containing glass wool (F) to remove any large droplets of water carried by the gas stream. Any remaining water vapour was removed using a drier (Nafion) (International Science Consultants) (G). Dried gas was subsequently passed through a 6-port switching valve (Alltech Associates Inc.) (H) and into a sample loop coiled to approximately 5 cm in diameter. The sample loop was held in a liquid nitrogen dewar (I) and suspended approximately 2 cm above the liquid nitrogen (J). The temperature of this headspace was maintained at  $-150 \pm 5^\circ\text{C}$  using an automated temperature control unit (Dunstaffnage Marine Laboratory) (K). The sample purge time depended upon the volume of the sample. Purge times for  $<50 \text{ cm}^3$  samples were 18 min, whilst larger samples ( $50$  to  $150 \text{ cm}^3$ ) took

~30 min. Once all the sample had been purged and concentrated in the sample loop, the 6-port rotary valve was adjusted to connect to the analytical equipment. The sample loop was then heated to  $>80\text{ }^{\circ}\text{C}$ , by submerging the loop in hot water.

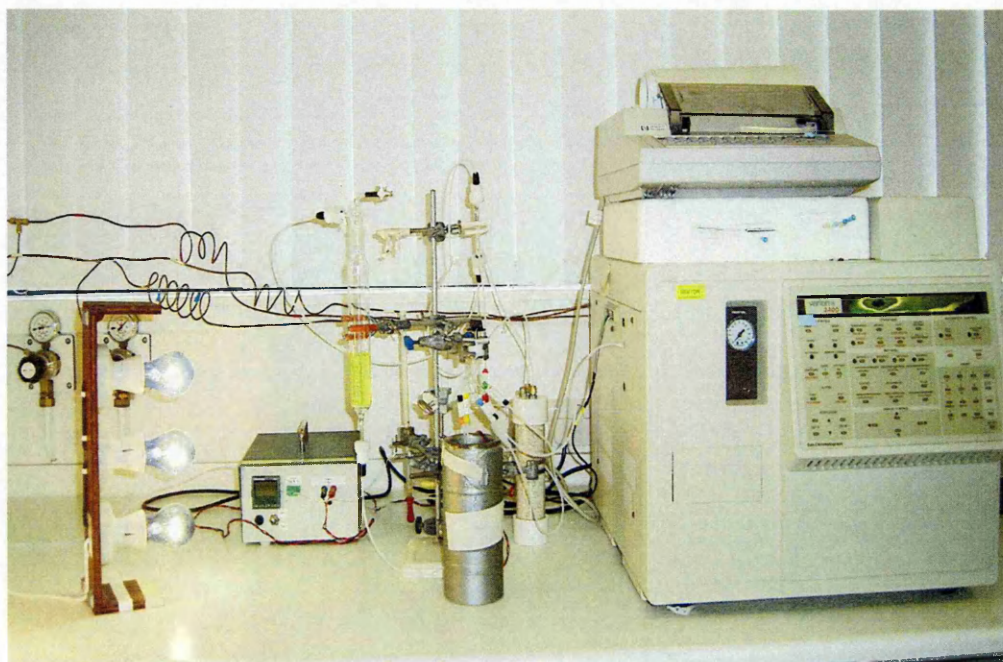


**Figure 2.1** Apparatus for the measurement of DMS. The sample preparatory system consisted of: A: oxygen-free nitrogen purge gas; B: purge chamber; C: porous frit; D: bubble meter; E: sample inlet with luer valve; F: glass tubing with glass wool; G: nafion drier surrounded by molecular sieve; H: 6-port valve; I: dewar; J: liquid nitrogen; K: temperature control unit; L: Gas Chromatograph Unit; M: Flame Photometric Detector; N: Chromasil 330 packed column; O: integrator.



### *Sample analysis*

The analytical equipment consisted of a Varian 3300 gas chromatograph (L) fitted with a flame photometric detector (FPD: dual flame detector with 365 nm filter) (M) and a Chromasil 330 column (Teflon 4.6 ft x 1/8" o.d.) (N) (Fig. 2.2). Oxygen-free nitrogen was used as the carrier gas at a flow rate of  $30 \text{ cm}^3 \text{ min}^{-1}$ . The gas chromatograph was operated with a temperature programme of 40-85 °C and DMS was eluted at 4.1 min. The detector output was monitored on a Hewlett-Packard 3390A reporting integrator (O). The detection limit was  $0.05 \text{ nmol dm}^{-3}$  sulphur.



**Figure 2.2** Photo taken of the gas chromatograph and the purge-and-trap set-up, being used for DMSO analysis.

#### **2.1.2 Analytical procedure for sulphur compounds**

Biogenic sulphur analysis was conducted for DMS, DMSP (both the particulate and dissolved fraction) and DMSO (the particulate and dissolved fractions). When

appropriate, the different components were measured sequentially from the same sample, though the analytical procedure is described for each compound separately below.

*i) DMS*

For DMS measurements, the sample was slowly drawn into a glass syringe and used to gently rinse the syringe before being expelled. Subsequently, a 1-50 cm<sup>3</sup> sample was drawn into the syringe whilst ensuring a limited headspace was maintained to prevent any de-gassing of DMS. The sample was then gently filtered using minimal pressure through a 25 mm AP25 depth filter (Millipore, UK) into the purge chamber of the sample preparation system and analysed as previously described.

*ii) DMSP dissolved (DMSPd)*

After the removal or analysis of DMS, samples were prepared for DMSPd analysis by transferring a 2-50 cm<sup>3</sup> sub-sample to either a 20 or 60 cm<sup>3</sup> glass crimp-top vial depending upon sample volume. The vial contained 2 cm<sup>3</sup> 10 M NaOH and was filled to the top with distilled water before being sealed with aluminium crimp seals with Pharma-Fix<sup>TM</sup> liner (Alltech Associates Inc.) with no headspace. Vials were stored in the dark for a minimum of 12 hours to allow DMSP to be completely hydrolysed to DMS before analysis. For quantification, the seal was removed and an aliquot added to the purge chamber as previously described for DMS.

*iii) DMSP particulate (DMSPp)*

Sample volumes of 2 to 20 cm<sup>3</sup> were gently drawn into glass syringes and gently filtered onto Millipore AP25 depth filters for analysis of DMSPp. The filter was subsequently added to a 20 cm<sup>3</sup> glass crimp vial with 2 cm<sup>3</sup> 10 M NaOH and distilled water. The vials

were immediately filled to the top with distilled water and sealed gas-tight using an aluminium seal with Pharma-Fix<sup>TM</sup> liner (Alltech Associates Inc) with no headspace. Vials were stored in the dark for a minimum of 12 hours to allow DMSP to be completely hydrolysed to DMS before analysis. For quantification, the seal was removed and an aliquot added to the purge chamber as previously described for DMS.

*iv) DMSO dissolved (DMSOd)*

The analysis of DMSO was performed indirectly, via enzymatic reduction of DMSO to DMS (Hatton 1994) and subsequent quantification of the DMS as previously described. DMSO reductase was purified (Dunstaffnage Marine Laboratory) from the purple 'non-sulphur' bacterium *Rhodobacter capsulatus* mutant strain H123.

Reduction of DMSO to DMS was achieved using a reducing solution which contained 25  $\mu\text{g cm}^{-3}$  DMSO reductase, 30 mM EDTA and 540 mM FMN. Depending upon the sample, 2 to 3  $\text{cm}^3$  of the reducing solution was added to the purge chamber using a disposable pipette and the sample subsequently injected through a 2-way luer valve into the purge chamber. The sample and reducing solution mix rapidly due the turbulence created by the gas flow 60  $\text{cm}^3 \text{min}^{-1}$  oxygen-free nitrogen. The purge chamber was illuminated (3 x 60 W daylight bulbs, RS Components). Under these conditions, EDTA forms radicals which reduce FMN to FMNH<sub>2</sub> (Massey et al., 1978), which acts as an electron donor to DMSO reductase (McEwan et al., 1985) catalyzing the reduction of DMSO to DMS. DMS is then quantified by gas chromatography as mentioned above.

*v) DMSO particulate (DMSOp)*

Sample volumes of 2 to 40  $\text{cm}^3$  were gently drawn into glass syringes and gently filtered onto Millipore AP25 depth filters for analysis of DMSOp following the method of Hatton

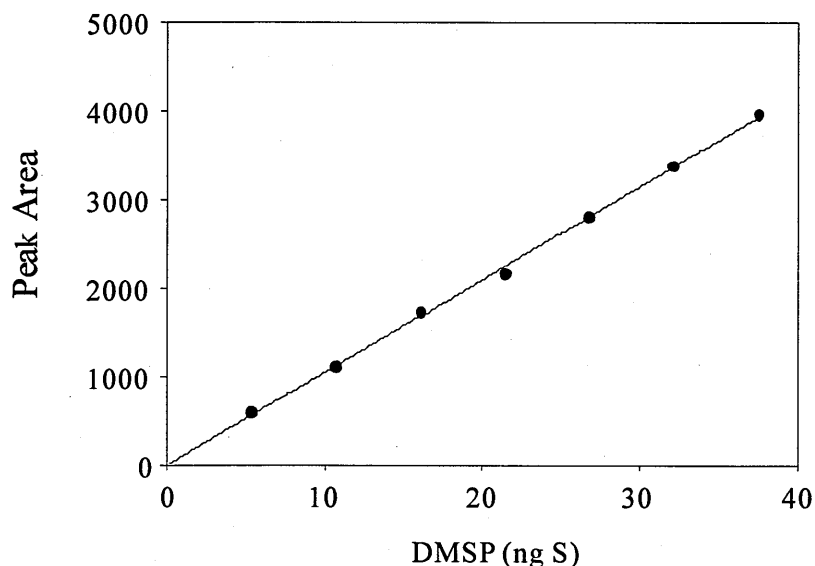
2002b. The filter was subsequently added to a 20 cm<sup>3</sup> glass crimp vial with 2 cm<sup>3</sup> 10 M NaOH, and 18 cm<sup>3</sup> 50 mM Tris buffer [pH 8.0]. The vials were immediately sealed gas-tight using an aluminium seal with Pharma-Fix<sup>TM</sup> liner (Alltech Associates Inc) with no headspace. Vials were stored in the dark for a minimum of 12 hours prior to analysis. Analysis was usually performed within 1 week of preparation (Simo 1998).

The strong alkali has the dual effect of breaking cells opening, thereby releasing DMSOp into the dissolved fraction and also hydrolysis of cellular DMSPp to DMS. To avoid contamination by DMS, after the vials were opened, samples were purged for 10 min at 150 cm<sup>3</sup> min<sup>-1</sup> (Darroch, 2003). It is also essential to have a final pH of 6.5 to 7.5 for the optimal functioning of the enzyme (Hatton et al., 1994) and therefore hydrochloric acid was added to adjust the pH. Analysis was then performed as described for DMSOd.

### 2.1.3 Calibration of the gas chromatograph for DMSP and DMSO

#### *i) DMSP*

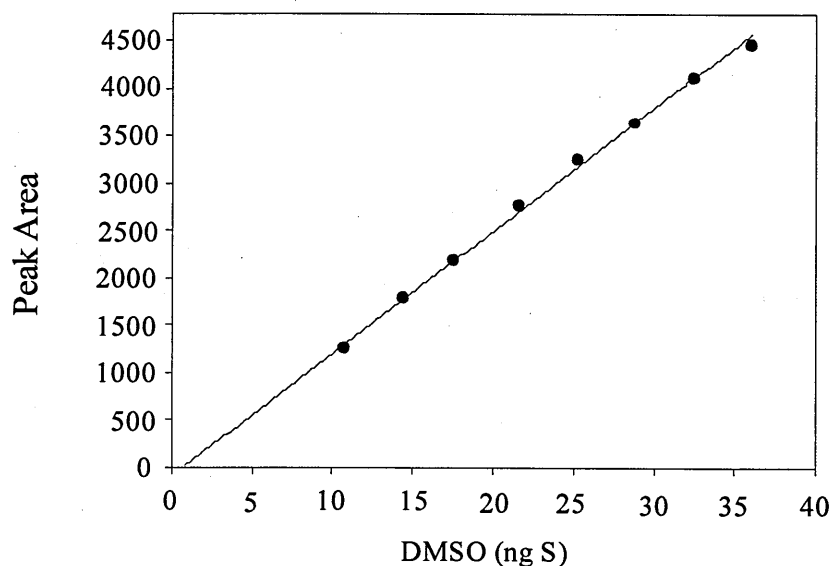
DMSP is readily cleaved by the strong alkali at room temperature to produce DMS and acrylic acid in a 1:1 ratio (Challenger 1959). This reaction is used to produce the DMS standard curve and calibrate the gas chromatograph. Stock concentrations of DMSP (University of Groningen, Netherlands) were used to prepare a working standard of 2.6 ng S cm<sup>-3</sup>. A range of volumes were injected into the purge chamber containing 2 cm<sup>3</sup> 10 M NaOH to produce a standard curve (Fig. 2.3).



**Figure 2.3** Typical calibration curve for DMSP:  $y=105.4x - 3.48$ ;  $r^2=0.998$

*ii) DMSO*

DMSO standards were prepared using a working stock concentration of  $3.58 \text{ ng S cm}^{-3}$  from DMSO 99.5% (Sigma-Aldrich, UK). Due to the ubiquity of DMSO in the environment, stringent precautions must be taken to avoid contamination. All reagents were made from fresh purified Milli-Q water and prepared every 1 to 2 days. Reagents were stored at  $4^\circ\text{C}$  in the dark to prevent an increase of DMSO during storage and all glassware was kept clean and separate from general laboratory use. A range of volumes were injected into the purge chamber containing  $2 \text{ cm}^3$  reducing solution (section 2.1.3) to produce a standard curve (Fig. 2.4).



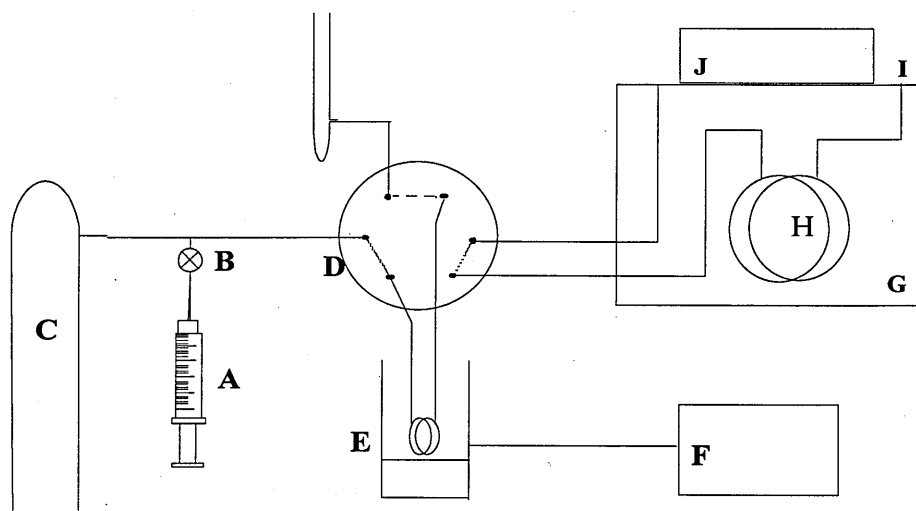
**Figure 2.4** Typical calibration curve for DMSO:  $y = 124.45x - 58.5$ ;  $r^2 = 0.997$

The calibration of the GCs and an understanding of the analytical precision associated with the instrument are highly important in being able to discriminate between genuine biogeochemical trends and those resulting from sampling and analytical associated effects. When running samples, the calibration of the system was checked daily using the DMSP and the DMSO standards. A linear response was always observed in the range of concentrations analysed. A full calibration of six standards took approximately two hours. The precision for each standard based on a triplicate run of the higher concentration standards was better than 0.4 %. A calibration check for instrument drift over a two month period from February to March 2005 (when the grazing experiments were run (Chapter 4)) revealed a 2.14 % drift in the detector response.

## 2.2 Methane analysis

### *Analytical equipment*

Methane analysis was performed by gas chromatography using headspace equilibration (Fig. 2.5) (Johnson et al., 1990; Upstill-Goddard et al., 1996). Samples (A) were introduced into the preparatory system via a 2-way valve (B). The sample passed directly into a nitrogen gas stream (C) ( $60 \text{ cm}^3 \text{ min}^{-1}$ ). The gas was subsequently passed through a 6-port switching valve (D) (Alltech Associates Inc.) and into a coiled sample loop packed with Porapak Type Q 80-100 (Alltech Associates Inc.) suspended above liquid nitrogen within a small dewar (E). The temperature of this headspace was maintained at  $-150 \pm 5$  °C using an automated temperature control unit (F) (Dunstaffnage Marine Laboratory). After a sample purge time of 2 min, the 6-port rotary valve was adjusted to connect to the analytical equipment. The sample loop was then heated to  $>80$  °C, by submerging the loop in hot water. The gas chromatograph was operated with a temperature programme of 45-85 °C and methane was eluted at 1.3 min. The detector output was monitored on a Spectra-Physics SP4290 reporting integrator (J). Precision for replicate samples was within 1 % and detection limit was 0.1 nmol. The analytical system consisted of Varian 3400cx GC (G) fitted with a 6 ft x 1/8" s/s packed molecular sieve column (H) and a flame ionisation detector (I).

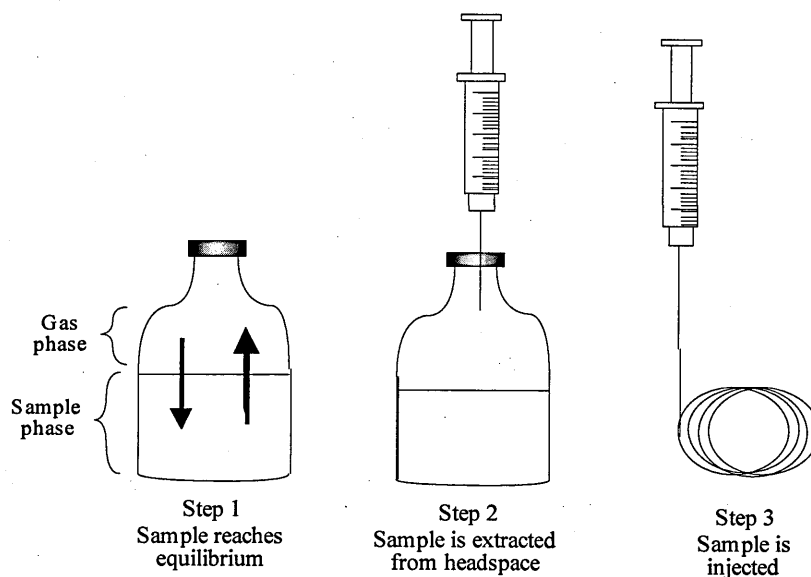


**Figure 2.5** Apparatus for the measurement of methane. The sample preparatory system consisted of: A: syringe with sample; B: 2-way valve; C: nitrogen gas stream; D: 6-port valve; E: liquid nitrogen; F: temperature control unit; G: Gas Chromatograph Unit; H: Chromasil 330 packed column; I: Flame Photometric Detector; J: integrator.

### *Analytical procedure for methane analysis*

Two types of sample were analysed for methane concentrations: the headspace of slurry experiments to investigate changes in methane production over time; and methane concentrations in seawater. To measure methane in seawater, samples were decanted into 60 cm<sup>3</sup> glass vials with the exclusion of all air and sealed with aluminium butyl rubber crimp seals. No preservative was used as samples were analysed within 2 hours of collection. In the laboratory, a 6 cm<sup>3</sup> headspace of oxygen-free nitrogen was introduced to the glass vial using a hypodermic needle and seawater was expelled via a second needle (Fig. 2.6). Samples were vigorously shaken and left to equilibrate between the gas and liquid phase fully at ambient temperature for 2 hours. Samples of headspace (typically 1-2 cm<sup>3</sup>) were then analysed on the GC.





**Figure 2.6** Gas syringe system used in the headspace analysis for methane

For the analysis of headspace methane in the anaerobic slurry microcosms (Chapter 6), a similar procedure as described for seawater samples (and Fig. 2.6) was followed. The sample vial volume was 20 cm<sup>3</sup> with a headspace of 3 cm<sup>3</sup>. The concentrations of methane measured in the headspace of slurry experiments were not converted to reflect the water-phase equilibrium as the study compared unamended and amended samples. In contrast, the methane concentration in seawater samples was calculated from the headspace concentration using the calculations of Kampbell and Vandegrift (1998) and also referred to by the US Environmental Protection Agency (2001).

**Equation 2.1** Concentration of methane in seawater phase (mg/L):

$$\text{Concentration in seawater sample (mg/L)} = \left( \frac{\text{Conc (ppmv)} * n_g * \text{MW}}{\text{Henry's Law constant}} \right) * 1000$$

Conc (ppmv) is the partial pressure ( $p_g$ ) of the methane in the sample (expressed as a decimal equivalent) calculated from linear regression equation of the standard curve;

Henry's Law constant is 37,600 (at 20 °C); MW is the molecular weight of methane;  $n_g$  represents the moles of gas (1 L of water equals 55.5 g-moles)

**Equation 2.2** Concentration of methane in headspace phase (mg/L):

$$\text{Concentration in headspace (mg/L)} = \frac{A_i * \text{Density factor}}{\text{Vol}_w}$$

$\text{Vol}_w$  represents the volume of the water sample ( $\text{dm}^3$ ) (which is the sample bottle volume minus the headspace volume).  $A_i$  represents  $\text{cm}^3$  of  $\text{CH}_4$  in the headspace and is calculated by:

$$\text{Equation 2.2a } A_i = \text{Conc (ppmv)} * \text{Vol}_h$$

$\text{Vol}_h$  represents the volume of the headspace ( $\text{cm}^3$ ).

The density factor is calculated by:

**Equation 2.2b** Density factor of analyte in headspace:

$$\text{Density factor of analyte} = \frac{\text{MW} * 273 \text{ (K)}}{22.4 \text{ (L/mole)} * \text{Temp (K)}}$$

Whereby Temp (K) represents the temperature of the sample in Kelvin.

The final concentration in the original water sample is the sum of both phases. Therefore, the Total Sample Concentration (mg/L) = Concentration in water phase (Equation 2.1) + Concentration in headspace phase (Equation 2.2)

*Example calculation for methane.*

From the analysis of the sample, a peak area was determined. This peak area was used in the equation for the linear regression of the calibration curve to provide the partial pressure. Parameters used in this example are as follows: the sample peak area was 24488, Henry's Law constant was 37,600 (at 20 °C), the sample temperature was 20 °C (293 K), the bottle volume was 60 cm<sup>3</sup> and the headspace volume was 6 cm<sup>3</sup>.

Using the calibration standard response curve of  $P_g = (159675)x - 8045$  and a peak area of 24488:

$$\begin{aligned} P_g &= (24488 + 8045)/159675 \\ &= 0.2037 \text{ nmol CH}_4 \\ &= 0.00000456 \text{ ppmv} \end{aligned}$$

Then using the previous equations:

From Equation 2.1:

$$\begin{aligned} \text{Concentration in} \\ \text{seawater sample (mg/L)} &= \left( \frac{0.00000456 * 55.5 * 16}{37,600} \right) * 1000 \\ &= 0.00011 \text{ mg CH}_4 \text{ per dm}^{-3} \text{ seawater} \end{aligned}$$

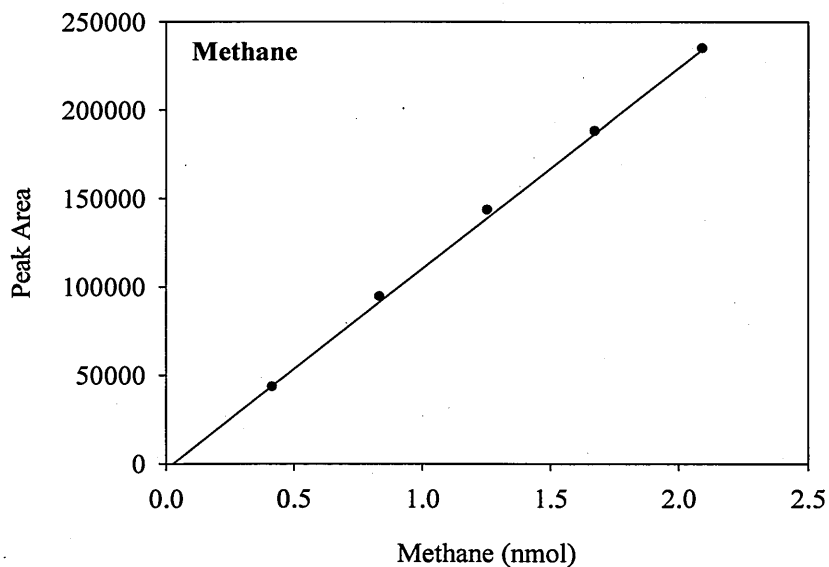
From Equation 2.2:

$$\begin{aligned} \text{Concentration in} \\ \text{headspace (mg/L)} &= \frac{0.00002736 * 6}{0.054} \\ &= 0.0003 \text{ mg CH}_4 \text{ per dm}^{-3} \text{ headspace} \end{aligned}$$

Therefore, total concentration in the original sample was 0.00041 mg CH<sub>4</sub> per dm<sup>-3</sup> seawater, which converts to 25.6 nmol CH<sub>4</sub> per dm<sup>-3</sup>.

### Calibration of the gas chromatograph

A methane standard (Alltech Associates, UK) of 10 ppm in nitrogen was used as the methane standard. To produce the methane standard curve and calibrate the gas chromatograph a range of volumes (0 to 10 cm<sup>3</sup>) were used (Fig. 2.7).



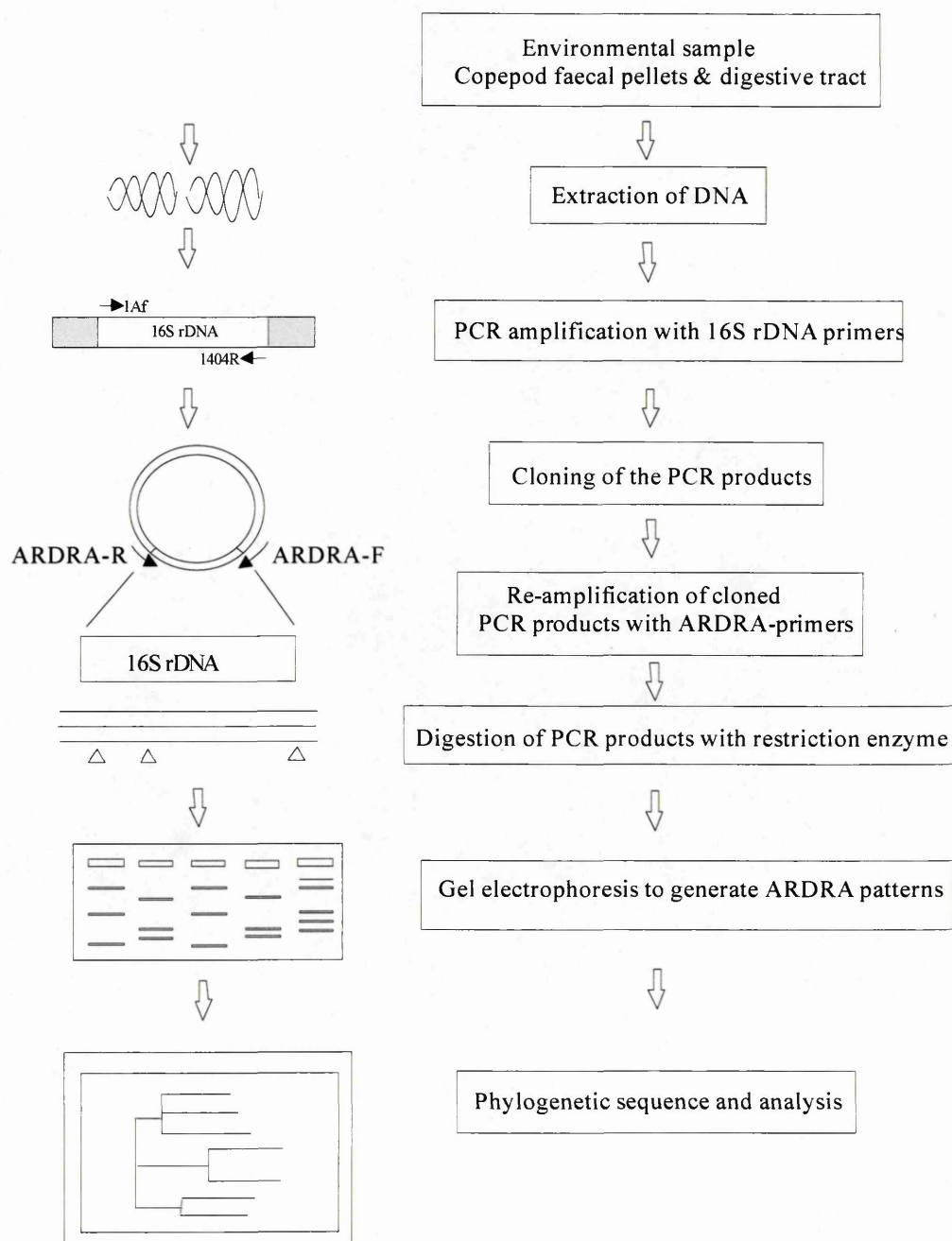
**Figure 2.7** Typical calibration curve for methane:  $y = 113828x - 2656.6$ ;  $r^2 = 0.99$

### 2.3 Overview of molecular biology

Small subunit 16S ribosomal RNA (16S rRNA) was used to characterise the Euryarchaeal community. The rRNA gene is an important phylogenetic marker (Woese et al., 1990) and is a useful tool for analysing natural microbial populations (Olsen et al., 1986; Pace et al., 1986). Among the three existing ribosomal RNA (5S, 16S/18S and 23S/28S), the 16S rRNA molecule is the most widely used marker (Head et al., 1998). Figure 2.8 outlines the method of obtaining phylotypes from the environment using polymerase chain reaction (PCR) and the different steps to the procedure discussed below:

1. The essential starting point is the effective extraction of nucleic acids from environmental samples. There are a number of empirically derived methods for nucleic acid extraction that have become acceptable. Table 2.1 describes the two different methods used in this study. The extraction process has been characterised into distinct sections: pre-treatment of the sample; cell lysis; nucleic acid isolation and nucleic acid purification. The pre-treatment step refers to procedures undertaken prior to cell lysis *e.g.* the washing of soils and sediments with an alkaline phosphate solution to remove extracellular nucleic acids (Tsai and Olson, 1991). The lysis of cells can be divided into chemical, enzymatic, mechanical and other types. In most procedures, a combination of the three techniques are usually employed *e.g.* the combination of glass beads with sodium dodecyl sulphate (SDS) solution. The isolation of DNA classically involves an alkaline phosphate buffer (Sambrook et al., 1989) which has been used effectively in extractions from environmental samples. After nucleic acid isolation, it is usually necessary to purify the product to remove contaminants such as carbohydrates, humic acids and proteins. Proteins can be removed using phenol/chloroform extraction, hydroxyapatite separation

and caesium chloride centrifugation (Purdy, 2005). The final step is the precipitation of nucleic acids. The most common procedure is ethanol precipitation (Sambrook et al., 1989) although isopropanol can also be used as it also reduces contamination by other PCR inhibitors in DNA extracts.



**Figure 2.8** Flowchart of the procedure adopted for the analysis of DNA extracted from environmental samples

Table 2.1 Nucleic acid extraction techniques used in this study highlighting the different procedures used in each process

Reference	Henckel (1999)	Van der Maarel (1997)
1. Sample pre-Treatment		
2. Lysis Chemical; Mechanical; Enzymatic	Cells lysed with cell disruptor. Solution contains: 0.1mm beads; 800µl NaPh 120mM pH 8; 260µl SDS solution (10% SDS; 0.5 M Tris-HCl pH 8.0; 0.1 M NaCl)	Lysis Buffer: (per 1.8ml) 5% (wt/vol) sucrose; 50mM EDTA; 5mM Tris-Hcl pH 8; 1M guanidinium thiocyanate; 0.4mg lysozyme)  Followed by: 67µl SDS solution for 30 min at 37°C 1 mg Proteinase K for 3-4 h at 55°C.
3. Nucleic acid isolation	Resuspension in 700µl phosphate buffer	Nucleic acids were extracted with phenol-chloroform-isoamylalcohol (25:24:1)
4. Nucleic acid purification (Remove protein, carbohydrates, humic & fulvic acids)	0.4 volume 7.5 M ammonium acetate	
5. Nucleic Acid precipitation	0.7 volume isopropanol with 70% ethanol	900µl ethanol (96%) and 7.5 M ammonium acetate
6. Resuspension of DNA	Resuspend DNA in 200µl of TE buffer	Resuspend in 100µl of TE buffer.

2. The extracted DNA can then be subject to PCR amplification of the 16S rRNA gene. The methanogens were the one of the first microbial groups to have their taxonomy based on phylogeny inferred from 16S rRNA sequence divergence (Balch 1979). Since then the database of 16S rRNA sequences has been used as a basis for the design of 16S rRNA-targeted primers. Primers allow the identification of amplifications or identification of sequences from a Euryarchaea domain down to individual genera or species level. Table 2.2 lists the primers used in this study. The resulting snapshot of community diversity will

depend upon the specificity of the primers and the efficiency with which the rRNA genes are amplified.

**Table 2.2** Primers used in PCR reactions during this study. References: 1: Embley (1992); 2: Lane (1990); 3: Messing (1983); 4: Green (2004).

Name	Specificity	Sense	Primer sequence 5'-	Ref.
1A	Archaea	Forward	TCYGKTTGATCCYGSCRAG	1
1100A	Archaea	Reverse	TGGGTCTCGCTCGTTG	1
1404A	Archaea	Reverse	CGGTGTGTGCAAGGRGC	1
27F	Bacteria	Forward	AGAGTTTGATCMTGGCTCAG	2
1492R	Bacteria	Reverse	ACGGCTACCTTGTTACGACTT	2
M13	pGemT Vector-based	Forward	GTTTTCCCAGTCACGAC	3
M13	pGemT Vector-based	Reverse	CAGGAAACAGCTATGAC	3
ARDRA-F	pGemT Vector-based	Forward	GCCATGGCGGCCGCGGGAATT	4
ARDRA-R	pGemT Vector-based	Reverse	AGGCGGCCGCGAATTCCTAG	4

3. Subsequent to amplification, the PCR product is cloned using standard procedures. A number of community fingerprinting techniques exist for analysis of microbial diversity within a sample. These techniques include denaturing gradient gel electrophoresis (DGGE) and restriction fragment length polymorphism (RFLP) and they offer a compromise between the number of samples processed and the information obtained about community diversity. RFLP analysis of community rRNA genes was used in this study. RFLP produces rRNA gene fragments that may be specific for different community members which allows individual community members to be qualitatively identified in environmental samples using gel electrophoresis.

4. The most commonly used form of comparative rRNA gene analysis involves construction of phylogenetic trees. This involves the careful and highly critical alignment of the RNA sequences followed by phylogenetic analysis. A range of methods exist for inferring phylogenetic trees such as pairwise distance, parsimony and maximum likelihood

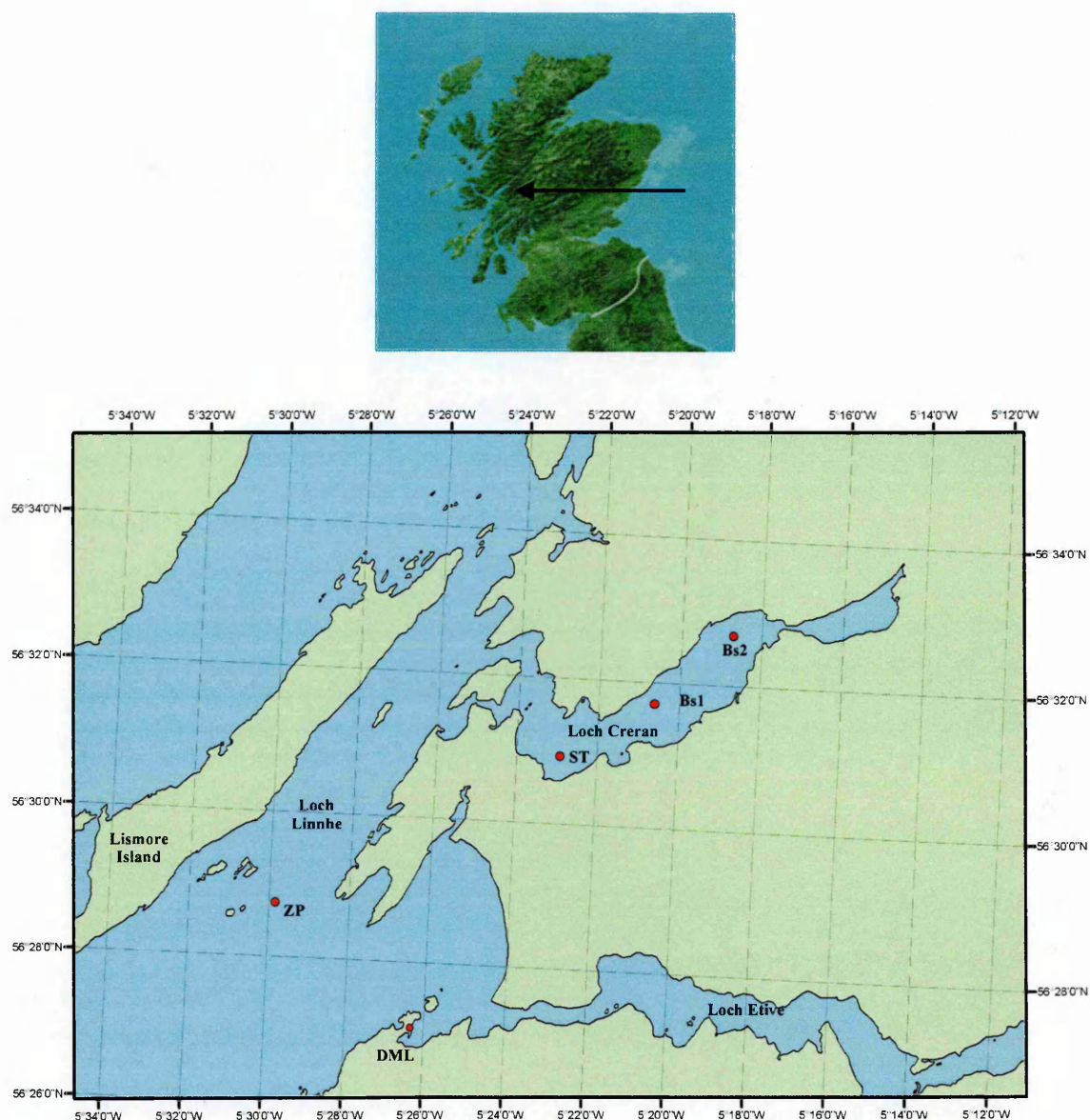


(Swofford et al., 1996). To assess the reliability of the branching pattern within the tree topology, bootstrap analysis is performed. This involves the construction of new sequences sets by resampling with replacement sites of the original set, building a tree for each new set, and calculating the percentage of times a cluster reappears in the bootstrap replications.

## 2.4 Fieldwork

The fieldwork in this thesis was conducted in the nearby Scottish coastal environment using Dunstaffnage Marine Laboratory's *RV Seol Mara* and small boats. The fieldwork included analysis of seawater samples, deployment of sediment traps and benthic samplers and isolation of phytoplankton and zooplankton for culturing and analysis. Figure 2.9 highlights the location of the respective sampling occasions in comparison with Dunstaffnage Marine Laboratory.

The Scottish west coast is penetrated by numerous sea-lochs that are a product of glaciation. Loch Creran is one such sea-loch, situated approximately 15 km north of Oban. Loch Creran is referred to as the 'archetypical' Scottish sea loch due to its dimensions, freshwater input and tides (Landless and Edwards, 1976). The loch is a fjordic type consisting of a glacially over-deepened valley and separated from the open sea by a sill of minimum 5 m depth in a 0.2 km wide channel (Tett and Wallis, 1978). Loch Creran consists of an upper basin (3 km by 0.8 km) and a lower basin (11 km by 1.5 km) with a maximum depth of 58 m (Milne, 1972). The deepest part of the loch is situated near the seaward end of South Shian. Here a broad depression occurs in the bed of the loch for a distance of 6 km to the east of South Shian.



**Figure 2.9** Location of sampling sites. (DML: Dunstaffnage Marine Laboratory; ZP: Zooplankton trawls (Chapter 4, 7 & 8); ST: Sediment trap (Chapter 5, 6 & 7); Bs1 & Bs2: Benthic sampling (Chapter 6). Details of surface seawater samples are provided in Chapter 5.

Loch Creran connects to Loch Linnhe which is a large body of water separating Ardnamurchan from the rest of Argyll. Loch Linnhe is located at the south western end of the Great Glen fault which traverses the highlands of Scotland and comprises the main section of the largest sea-loch system on the west coast of Scotland. The inner loch basin is surrounded by some of the highest mountains in Scotland and has a large freshwater input due to the extreme rainfall and large catchment area. The outer basin, which is shown in Figure 6, is more open than the inner basin, being 2-3 times wider, shallower (80m) and having a smaller input of freshwater (Taylor, 1997). The outer basin opens into the Firth of Lorne which represents a well mixed extension of the open continental shelf.



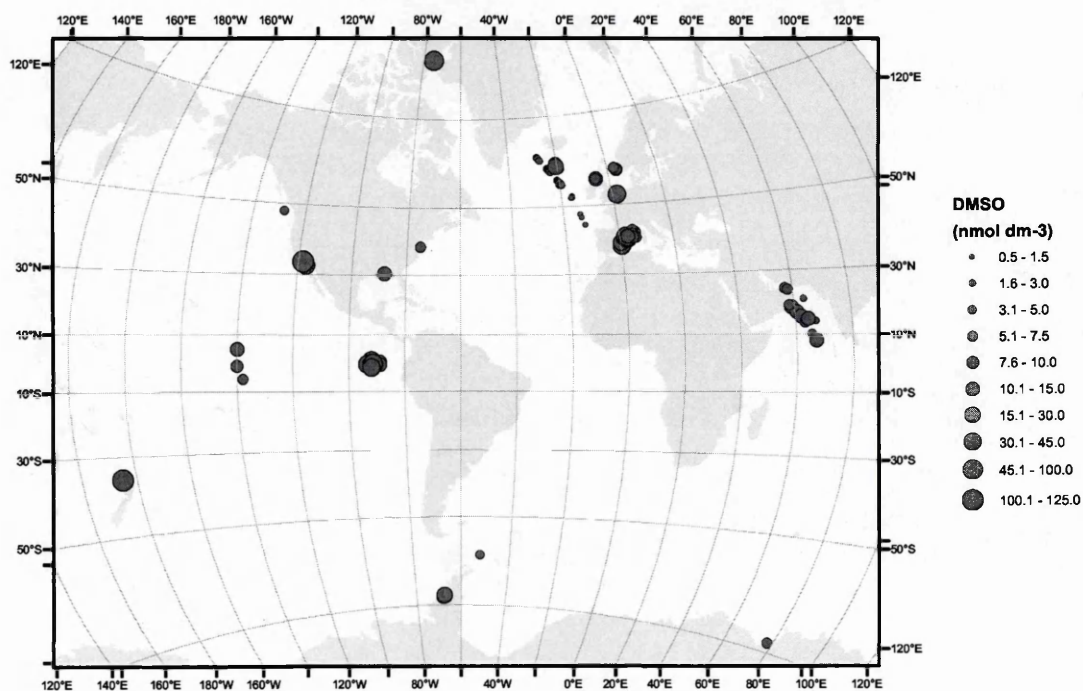
## **Chapter 3. The production of dimethylsulphoxide and dimethylsulphoniopropionate in laboratory cultures of marine phytoplankton**

### **3.1 Introduction**

The biogenic sulphur compounds dimethylsulphide (DMS), dimethylsulphoniopropionate (DMSP) and dimethylsulphoxide (DMSO) have received considerable attention in marine research, partly due to the influence of DMS on the Earth's climate (Charlson et al., 1987). Most research has focused on the marine environment because DMS is the most abundant volatile sulphur compound in the world's oceans (Liss et al., 1997). Although there is an increasing tendency to consider DMS, DMSP and DMSO as an inter-related group of compounds, there is an overwhelming larger body of knowledge on the oceanic distribution and dynamics for DMS and DMSP than for DMSO. This is largely due to the discovery of DMS and DMSP in seawater and marine phytoplankton prior to DMSO, but is also due to the intrinsic difficulties in the analysis of DMSO at nanomolar levels in seawater (Hatton et al., 1994; Simó, 1998).

DMSO occurs naturally in a range of environments including rainwater (Ridgeway et al., 1992); atmosphere (Berresheim et al., 1993); freshwater (Andreae, 1980); and also the marine environment. Concentrations of dissolved DMSO (DMSOd) in the marine environment are typically at the lower end on the nanomolar scale, though concentrations exceeding  $100 \text{ nmol dm}^{-3}$  have been recorded in the coastal and open Pacific (Lee and de Mora, 1996; Hatton et al., 1998) and in the coastal Arctic (Lee et al., 2001; Bouillon et al., 2002). Elsewhere in the marine environment, DMSOd concentrations of  $3.8\text{--}26 \text{ nmol dm}^{-3}$  were measured in the North Atlantic (Simó et al., 2000),  $2\text{--}17.5 \text{ nmol dm}^{-3}$  in the North

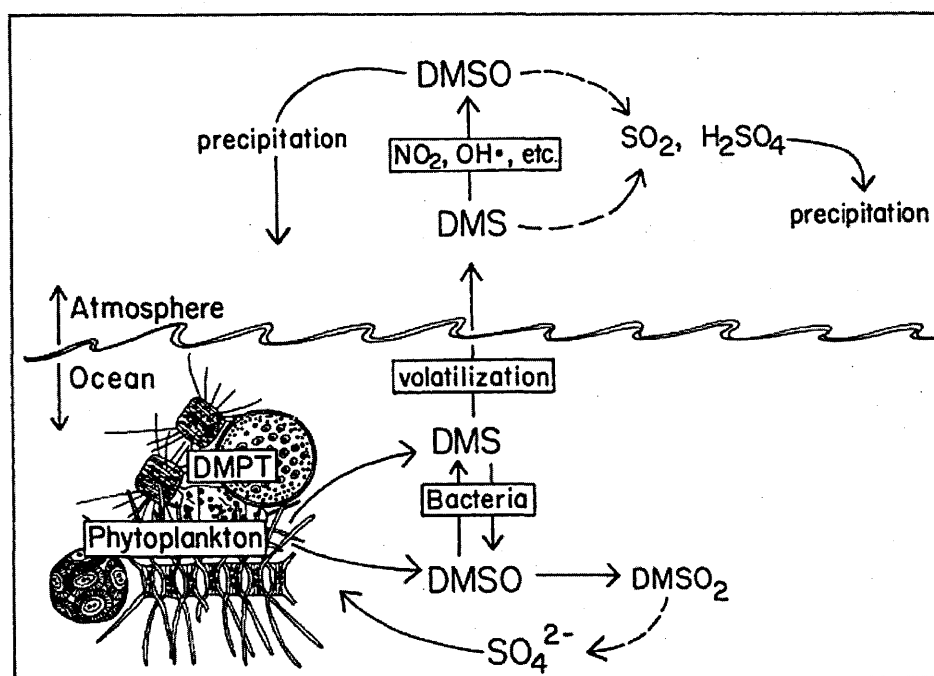
Sea (Hatton et al., 1996) and  $4\text{--}20\text{ nmol dm}^{-3}$  in the Pacific Ocean (Kieber et al., 1996). A compilation of field-based DMSO measurements is provided in Figure 3.1 and is also reported by Riseman and DiTullio (2004), and Simó and Vila-Costa (2006). Based on this growing dataset of DMSO concentrations, it is thought that in comparison to other methylated sulphur species, concentrations of DMSOd in surface waters are usually equal to, or exceed those for DMS, and are more comparable to those for DMSPd. However, whereas DMS and DMSP are typically restricted to the euphotic zone, DMSOd has been recorded at concentrations exceeding  $1.5\text{ nmol dm}^{-3}$  at depths exceeding 1000 m in the Pacific Ocean and the Arabian Sea (Hatton et al., 1998; Hatton et al., 1999). Therefore, depth-integrated DMSOd can be the dominant DMS-related species throughout the water column.



**Figure 3.1** Location and levels of DMSO in surface waters collated from published data, from Hatton et al. (2004).

The possibility of a direct synthesis pathway for DMSO was considered quite early on in the history of marine research on the biogenic sulphur compounds (Andreae, 1980).

Andreae (1980) described the distribution of dimethylsulfoxide in marine and freshwaters and its association with algal cultures. Unfortunately, the methodology used was prone to contamination, and it took over a decade before methods were sufficiently refined (Hatton et al., 1994; Simó et al., 1996) to revisit some of this early work. However, the schematic diagram from Andreae's (1980) study has been reproduced in this Chapter (Fig. 3.2) as it is still highly relevant in current discussions on the role of dimethylsulphoxide in the biogeochemical cycling of methylated sulphur compounds and its relationship with both DMS and DMSP.



**Figure 3.2** Proposed cycle of methylated sulphur compounds in surface of ocean and in marine atmosphere, from Andreae (1980). (DMPT [(CH<sub>3</sub>)<sub>2</sub>S<sup>+</sup>CH<sub>2</sub>CH<sub>2</sub>COO<sup>-</sup>] is now commonly referred to as DMSP).

The biosynthesis of DMSO by algae was also suggested to account for diurnal variations in DMSO concentrations observed in New Zealand coastal waters (Lee and de Mora, 1996). Subsequently, DMSO was measured in phytoplankton cultures of the dinoflagellate *Amphidinium carterae* and the coccolithophore *Emiliana huxleyi* (Simó et al., 1998). The discovery of an intracellular DMSO pool was referred to as particulate DMSO (DMSOp) distinct from the ambient pool of DMSOd. The intracellular production of DMSOp occurred during the logarithmic growth of both algal species. The average cellular content of DMSOp was approximately 0.3 and 0.1 pg DMSOp cell<sup>-1</sup> for *A. carterae* and *E. huxleyi* and a DMSPp:DMSOp ratios of 25 and 8 for *A. carterae* and *E. huxleyi* respectively was reported.

The potential synthesis of DMSOp by algae has led to speculation on its intracellular functions. DMSO is a very effective cryoprotectant and widely used by the biotechnology industry for cell preservation (Yu and Quinn, 1994). Therefore DMSOp was considered to lower the freezing temperature of intracellular fluid (Lee and de Mora, 1999). However, the same authors more recently concluded that cellular concentration of DMSOp in Arctic ice algae was considered too low to have a significant influence in its own right on the freezing point depression of intracellular fluids (Lee et al., 2001). Another cellular function of DMSO could be as a free-radical scavenger (Diamond et al., 1997; Hooiveld et al., 2003) as it may offer the algal cell protection against reactive oxygen radicals produced during photosynthesis. In this capacity, DMSOp has been described as part of a cellular antioxidant system, whereby DMSPp, its cleavage products DMS and acrylate, and the oxidant products of DMSOp protect against oxidative stress (Sunda et al., 2002). Additional functions of DMSOp could be as an intracellular electrolyte modifier (Lee and



de Mora, 1999) and also a protein-stabilising agent providing protection against thermally induced denaturation (Brown et al., 1977).

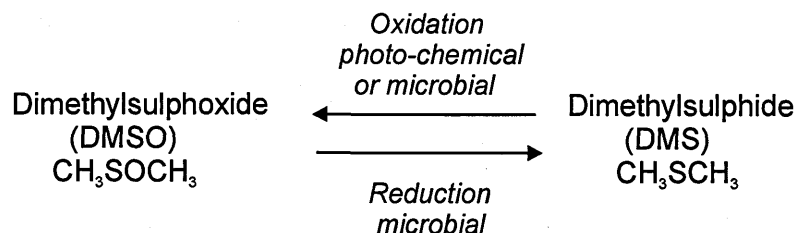
Since the confirmation of an intracellular pool of DMSOp by Simó et al. (1998), there have been increasing measurements made of DMSOp in the marine environment. DMSOp varied from 3-16 nmol dm<sup>-3</sup> in samples from the North Sea, North Atlantic and the North-West Mediterranean (Simó et al., 2000). Bouillon et al. (2002) reported DMSOp concentrations ranging from 0 to 17 nmol dm<sup>-3</sup> in a North Water Polyna Study which were exceeded by DMSOd concentrations of 13.1-106 nmol dm<sup>-3</sup>. The highest levels of DMSOp recorded so far were in Saguenay Fjord, Quebec, where DMSOp concentrations of  $\leq 110$  nmol dm<sup>-3</sup> were observed and on occasions exceeded levels of DMSPp. The concept that DMSOp is ubiquitous in the marine environment is supported by a recent analysis of DMSOp concentrations from 10 sites across the globe collected between 1995 and 2003 (Simó and Vila-Costa, 2006). DMSOp concentrations ranged from 1-40 nmol dm<sup>-3</sup> and were found to co-occur with DMSPp which ranged from 6-340 nmol dm<sup>-3</sup>. DMSOp accounted for an average of 20 % of intracellular dimethylated sulphur pool (DMSO+DMSP) which was reflected in an overall DMSPp:DMSOp ratio of 4.5 (Simó and Vila-Costa, 2006).

The correlation between DMSOp and DMSPp observed by Simó and Vila-Costa (2006) suggests that both compounds are closely linked intracellularly with a common origin in phytoplankton. However, the field-based measurements do not reveal whether DMSOp production is common throughout the phytoplankton taxa, or whether similar to DMSPp (Keller et al., 1989) it is preferentially produced by select algal groups. Neither is there

any indication that the DMSPp:DMSOp ratio varies among algal species, groups or size classes. It has been argued that DMSOp synthesis, relative to DMSPp production, may be more widespread amongst algal taxa (Lee et al., 1999). A greater prevalence of DMSOp production has also been advocated by Bouillon et al. (2002) based on the interpretation of DMSPp, DMSOp and chlorophyll *a* data in the North Water Polynya, Arctic Ocean. In their study, DMSPp did not correlate significantly with chlorophyll *a*, whereas DMSOp had a strong positive correlation with chlorophyll *a* and this finding was considered consistent with the notion of ubiquitous DMSOp production. The DMSPp and DMSOp:Chl *a* ratios were also subsequently used as evidence that DMSO production was more uniform in algal communities during an analysis on the Peruvian upwelling system (Riseman and DiTullio, 2004). Interestingly, both Bouillon et al. (2002) and Riseman and DiTullio (2004) suggested that fluctuating DMSPp concentrations were the dominant factor in determining DMSPp:DMSOp ratios. However, in contrast to the notion of ubiquitous DMSO production by algae, both Besiktepe et al. (2004) and Simó and Vila-Costa (2006) refer to unpublished laboratory work on algal cultures which suggests that DMSOp production occurs in the taxonomic-groups that produce DMSPp.

In addition to the algal production and release of DMSO, its presence in seawater can also arise from photochemical and microbiological processes (Fig. 3.3). The photo-oxidation of DMS to DMSO was first implied by Brimblecombe and Shooter (1986) who demonstrated that photolysis of DMS followed pseudo-first order reaction kinetics and that the mechanism involved singlet oxygen. More recently, field based observations have revealed that DMSO is a minor photolysis product and accounted for 14 % of DMS removal via photo-oxidation in the oligotrophic waters of the equatorial Pacific (Kieber et

al., 1996). The photo-chemical production of DMSO is supported by studies in a coccolithophorid bloom of the North Sea where DMSO accounted for <37 % of DMS removal (Hatton, 2002).



**Figure 3.3** Reduction-oxidation of DMSO and DMS in the marine environment.

The oxidation of DMSO can also be mediated by bacteria. The oxidation of DMS to DMSO was initially observed in cultures of obligate anaerobic bacteria (Zeyer et al., 1987; Visscher and van Gernerden, 1991a; Hansen et al., 1993) and it has subsequently been demonstrated that in addition to anaerobic oxidation, certain chemotrophic aerobic bacteria are similarly able to oxidise DMS to DMSO (Fuse et al., 1998). More recently, certain strains of the *Roseobacter* clade of the Proteobacteria  $\alpha$ -subclass have been shown to be able to aerobically oxidise DMS to DMSO (Gonzalez et al., 1999). Due to the abundance of Proteobacteria in the marine environment, particularly in blooms of DMSP-producing phytoplankton, these bacteria may be fundamental to the cycling of DMS and DMSO in seawater (Gonzalez et al., 2000; Zubkov et al., 2002). The biological reduction of DMSO to DMS has also been demonstrated, both anaerobically (Visscher and van Gernerden, 1991b; Jonkers et al., 1996; Vogt et al., 1997) and aerobically (Gonzalez et al. 1999). DMSO also represents a carbon source for microbes and the bacterial removal of DMSO without the production of DMS has been found to occur (de Bont et al., 1981; Suylen et al., 1986). The microbial turnover of DMSO in the marine environment has received very

little attention. Based on the incubation of natural seawater samples from the Mediterranean, the North Sea and the North Atlantic, Simó (2000) estimated microbial turnover times of 0.6 days for DMS, 1.3 days for DMSPd and 4 days for DMSOd.

To address the current lack of understanding of the relationship between DMSO and algal taxonomy, this work examined the production of DMSO in cultures of phytoplankton.

Specific objectives of this study are to investigate the

- i) relationship between algal taxonomy and DMSO;
- ii) the ratio of intracellular DMSP to DMSO;
- iii) the associated dissolved fractions of these compounds.

### 3.2 Materials and Method

Phytoplankton cultures were obtained from the Culture Collection of Algae and Protozoans (CCAP) or the personal collection of Dr. David Green (Table 3.1).

Dinoflagellates were grown in Kmin media (Leftley et al., 1987) whilst prymnesiophytes and diatoms were grown in f/2 media (Guillard and Ryther, 1962) (Table 3.2). Seawater for culturing was collected from Scottish coastal waters and aged prior to filtration and autoclaving. All equipment (glassware, tubing, filters) was autoclaved (121 °C for 20 min) before use. Cultures were grown in 300 cm<sup>3</sup> volumes in 500 cm<sup>3</sup> conical flasks and sealed with cotton bungs. All algal cultures were maintained at 14-15 °C on a 14:10 light:dark cycle.

**Table 3.1** List of phytoplankton species used in this study with the reference number from the Culture Collection of Algal & Protozoa. The initials DG represent strains which are from the personal collection of Dr. David Green. The abbreviations Cr and Sb refer to the isolation source of *P. micans* (Loch Creran and Santa Barbara respectively).

Dinoflagellates	Prymnesiophytes	Diatoms
<i>Prorocentrum micans</i> (Cr) 1136/19	<i>Isochrysis galbana</i> 927/1	<i>Skeletonema costatum</i> 1077/3
<i>P. micans</i> (Sb) DG	<i>Hymenomonas elongata</i> 961/3	<i>Thalassiosira pseudonana</i> 1085/12
<i>Gymnodinium simplex</i> 1117/3	<i>Emiliania huxleyi</i> DG	<i>Chaetoceros debilis</i> 1010/11
<i>Amphidinium operculatum</i> 1102/6	<i>Pleurochrysis carterae</i> 913/3	<i>Cyclotella cryptica</i> 1070/2
<i>Scrippsiella trochoidea</i> 1134/1	<i>Prymnesium parvum</i> 946/1D	<i>Phaeodactylum tricornutum</i> 1052/1A
<i>Gonyaulax spinifera</i> DG	<i>Phaeocystis</i> sp DG	<i>Bacillaria paxillifer</i> 1006/2
<i>Lingulodinium polyedrum</i> 1121/1	-	-
<i>Alexandrium tamarense</i> 1119/1	-	-
<i>Amphidinium carterae</i> 1102/1	-	-

To analyse cultures for DMSOp, DMSPp, DMSOd, DMSPd, with simultaneous cell counts, the culture flasks were gently stirred and sub-samples were taken by carefully decanting a small volumes ( $10\text{-}20\text{ cm}^3$ ) into a sterilised glass beaker ensuring no turbulence occurred. This was found to be a simple and sterile technique for repeatedly sub-sampling from multiple cultures of phytoplankton. Subsequently, a  $10\text{ cm}^3$  glass syringe was gently filled with the sample and a  $2\text{-}5\text{ cm}^3$  volume of sample was filtered through a AP depth filter. The filtrate was immediately purged for subsequent DMSPd analysis, whilst the filter was stored for future DMSPp analysis (Section 2.1.2). This procedure was repeated with fresh AP depth-filters for DMSOp and DMSOd analysis (Section 2.1.2). The remainder of the sample was used for cell counts. The volume of sample filtered depended on the concentration of DMSPp and DMSO within the different algal species and frequently a range of volumes were prepared for analysis. In all instances, the sample volumes were kept low and analysis was always conducted on algal cultures which were in early exponential growth phase.

Cell density was measured by manual counting using a Sedgewick-Rafter cell for dinoflagellates and a haemocytometer for prymnesiophytes and diatoms following fixation. All cells were fixed with 1 % Lugols, except for the coccolithophores where 2 % formalin and Rose Bengal was used. Cell volumes were determined from linear measurements of live cells (thirty replicates per species) with an Axiovision microscope. The nearest geometric shape was used to calculate cell volume as described in (Sun and Liu, 2003). The cell volumes for prymnesiophytes and dinoflagellates were calculated using the equation  $\pi d^2 l / 6$  ( $d$ =diameter;  $h$ =height and  $l$ =length) with the exception of *L.*

*polyedrum* ( $\pi d^2 l/12$ ) and *P. micans* ( $\pi d^2 h/12$ ), whilst the cell volumes for the diatoms were calculated using the equation  $\pi d^2 h/6$ .

An additional in-depth experiment was conducted separately by Angela Hatton using *Amphidinium carterae* grown in F/2+Se+L1 trace metals enriched natural seawater medium (Guillard and Hargraves 1993). Cells were grown in a 2 dm<sup>3</sup> glass culture flask sealed with a muslin bung, and maintained at 12 °C on a 14:10 light:dark cycle. Samples were collected every 2-3 days throughout the complete growth cycle (50 days). Each sample was analysed for DMS, DMSO, DMSOp, DMSPd and DMSPp using the procedure described in Section 2.1.2.

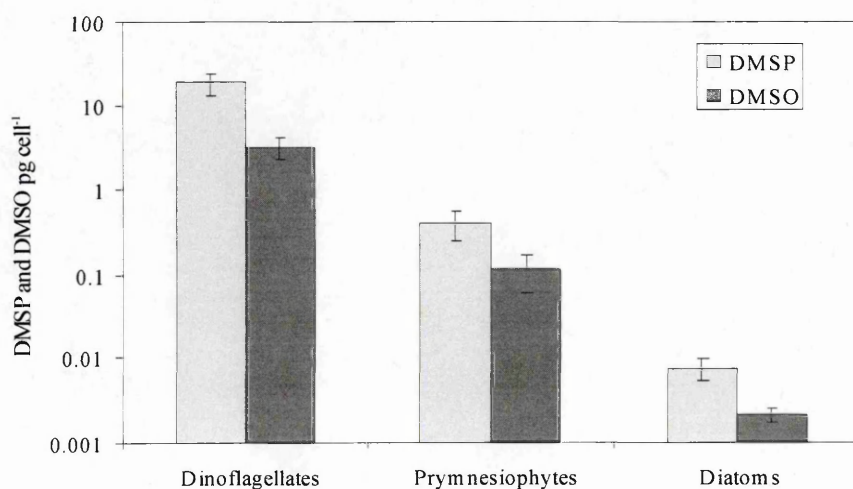
**Table 3.2** Media used for the culturing of phytoplankton

f/2 Medium	K minimum
<p><b>Stocks per litre</b></p> <p>(1) Trace elements (chelated)</p> <p>Na<sub>2</sub> EDTA 4.16 g</p> <p>FeCl<sub>3</sub>.6H<sub>2</sub>O 3.15 g</p> <p>CuSO<sub>4</sub>.5H<sub>2</sub>O 0.01 g</p> <p>ZnSO<sub>4</sub>.7H<sub>2</sub>O 0.022 g</p> <p>CoCl<sub>2</sub>.6H<sub>2</sub>O 0.01 g</p> <p>MnCl<sub>2</sub>.4H<sub>2</sub>O 0.18 g</p> <p>Na<sub>2</sub>MoO<sub>4</sub>.2H<sub>2</sub>O 0.006 g</p> <p>(2) Vitamin solution</p> <p>Cyanocobalamin (Vit B<sub>12</sub>) 0.0005 g</p> <p>Thiamine HCl (Vitamin B<sub>1</sub>) 0.1 g</p> <p>Biotin 0.0005 g</p> <p><b>Medium per litre</b></p> <p>NaNO<sub>3</sub> 0.075 g</p> <p>NaH<sub>2</sub>PO<sub>4</sub>.2H<sub>2</sub>O 0.00565 g</p> <p>Trace element stock solution (1) 1.0 ml</p> <p>Vitamin mix stock solution (2) 1.0 ml</p>	<p><b>Stocks per litre</b></p> <p>(1) Trace elements</p> <p>Na<sub>2</sub> EDTA 4.36 g</p> <p>Fe Cl<sub>3</sub>.6H<sub>2</sub>O 3.15 g</p> <p>ZnSO<sub>4</sub>.7H<sub>2</sub>O 0.022 g</p> <p>CoCl<sub>2</sub>.6H<sub>2</sub>O 0.01 g</p> <p>MnCl<sub>2</sub>.4H<sub>2</sub>O 0.18 g</p> <p>Na<sub>2</sub>MoO<sub>4</sub>.2H<sub>2</sub>O 0.006 g</p> <p>(2) Vitamin solution</p> <p>Cyanocobalamin (Vit B<sub>12</sub>) 0.0005 g</p> <p>Thiamine HCl (Vitamin B<sub>1</sub>) 0.1 g</p> <p>Biotin 0.0005 g</p> <p>(3) Na<sub>2</sub>SeO<sub>3</sub> 0.002 g</p> <p><b>Medium per litre</b></p> <p>NaNO<sub>3</sub> 0.075 g</p> <p>NaH<sub>2</sub>PO<sub>4</sub>.2H<sub>2</sub>O 0.00565 g</p> <p>Trace element stock solution (1) 1.0 ml</p> <p>Vitamin mix stock solution (2) 1.0 ml</p> <p>Na<sub>2</sub>SeO<sub>3</sub> stock solution (3) 1.0 ml</p>

### 3.3 Results

#### 3.3.1 DMSO and phytoplankton taxonomy

The intracellular concentrations of DMSPp and DMSOp are shown for representatives of the algal groups: dinoflagellates; prymnesiophytes; and diatoms (Table 3.3). DMSOp concentrations are highest in the dinoflagellates (average DMSOp concentration  $3.17 \text{ pg cell}^{-1}$ ); then prymnesiophytes (average DMSOp concentration  $0.11 \text{ pg cell}^{-1}$ ); followed by diatoms (average DMSOp concentration  $0.002 \text{ pg cell}^{-1}$ ) (Fig. 3.4). A one-way Analysis of Variance (ANOVA) for DMSPp and DMSOp demonstrated significant relationship between both DMSPp and DMSOp with the phytoplankton class (DMSPp:  $F_{2,20} = 7.75$ ;  $p=0.004$ ; DMSOp:  $F_{2,20} = 6.57$ ;  $p=0.007$ ). With the exception of one dinoflagellate species *G. simplex*, there is no overlap between the phytoplankton classes for either the DMSPp or the DMSOp values. These results demonstrate that as previously reported for DMSPp (Keller, 1989), DMSOp concentrations are correlated to taxonomic group.



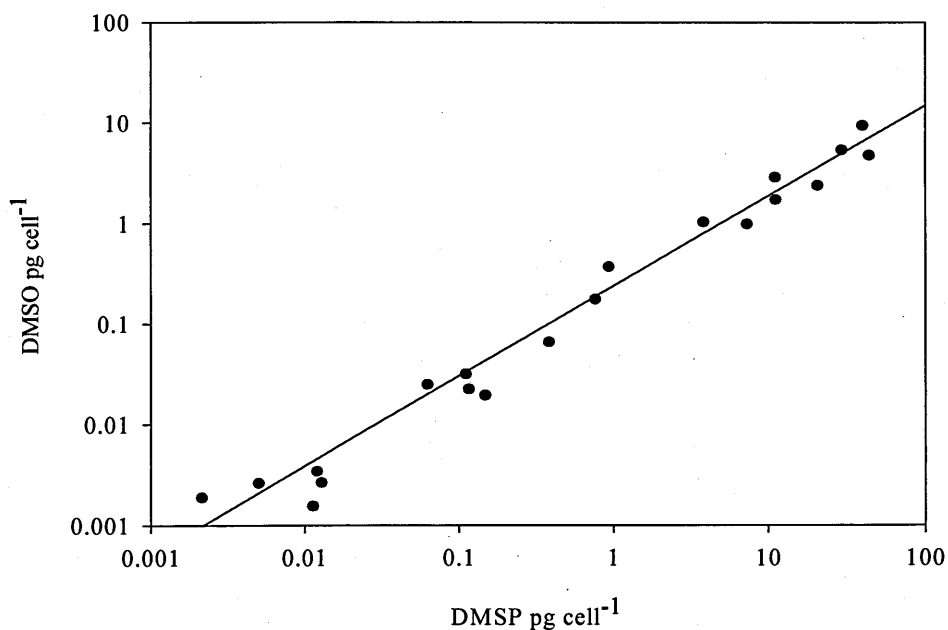
**Figure 3.4** Average (+SE) cellular concentrations of DMSOp and DMSPp for representatives of 3 major algal taxa (Table 3.2), plotted on a log scale. A one-way ANOVA showed significant relationship between both DMSPp ( $p=0.004$ ,  $n=20$ ) and DMSOp ( $p=0.007$ ,  $n=20$ ) and taxonomic class.



**Table 3.3** DMSOp and DMSPP concentrations associated with 3 major phytoplankton taxonomic groups. The algal cultures were analysed in early exponential growth phase. Intracellular concentrations of DMSO (DMSOp) and DMSPP (DMSPP) are provided as  $\text{nmol dm}^{-3}$  of culture and also as  $\text{pg cell}^{-1}$ . In addition, dissolved values for DMSO (DMSOd) and DMSPP (DMSPPd), cell volume and DMSPP:DMSO ratios are also highlighted.

Dinoflagellates		DMSPP $\text{nmol dm}^{-3}$	DMSOp $\text{nmol dm}^{-3}$	DMSPP $\text{pg cell}^{-1}$	DMSOp $\text{pg cell}^{-1}$	Cells per $\text{ml}$	Cell vol $\mu\text{m}^3$	DMSPP:DMSO per cell	DMSPPd $\text{nmol dm}^{-3}$	DMSOd $\text{nmol dm}^{-3}$
Prorocentaceae	<i>Prorocentrum micans</i> (Cr)	821.5	126.7	11.17	1.72	2,354	4000	6.48	31.7	12.5
	<i>Prorocentrum micans</i> (Sb)	1508.7	272.7	29.64	5.36	1,6229	4700	5.53	88.5	25.7
Gymnodiniaceae	<i>Gymnodinium simplex</i>	407.8	79.1	0.12	0.02	113,020	108	5.16	147.2	80.9
	<i>Amphidinium operculatum</i>	12041.2	2820.6	40.07	9.39	9,616	4015	4.27	186.8	230.6
Peridiniaceae	<i>Amphidinium carterae</i>	1461.5	393.3	3.84	1.03	12,187	1073	3.72	34.8	284.7
	<i>Scrippsiella trochoidea</i>	37167.5	3988.4	44.15	4.74	26,940	8167	9.32	199.3	353
Gonyaulaceae	<i>Lingulodinium polyedrum</i>	1289.9	173.5	7.31	0.98	5,646	44923	7.43	257.4	57.2
	<i>Gonyaulax spinifera</i>	973.7	112.1	20.77	2.39	1,500	13549	8.69	97.8	45.8
	<i>Alexandrium tamarense</i>	1769.5	458.3	11.09	2.87	5,104	17000	3.86	242.2	95
<b>Prymnesiophytes</b>										
Isochrysidales	<i>Isochrysis galbana</i>	191.6	75.8	0.063	0.025	98,000	53	2.53	21.3	4
	<i>Hymenomonas elongata</i>	1626.7	646.2	0.930	0.369	56,000	600	2.52	128.2	29.5
Coccosphaerales	<i>Emiliania huxleyi</i>	1543.9	440.8	0.110	0.031	448,000	55	3.50	10.5	36
	<i>Pleurochrysis carterae</i>	1067.8	246.2	0.759	0.175	45,000	120	4.34	28.3	72
Prymnesiales	<i>Prymnesium parvum</i>	4264.5	737.6	0.381	0.066	358,166	471	5.78	13.9	14.5
	<i>Phaeocystis</i> sp.	920.8	120.8	0.148	0.019	199,667	108	7.62	21.1	114
<b>Diatoms</b>										
Thalassiosiraceae	<i>Skeletonema costatum</i>	995.1	78.9	0.011	0.0015	$1.63 \times 10^6$	297	7.32	92.1	31.3
	<i>Thalassiosira pseudonana</i>	328.7	170.4	0.005	0.0026	$2.09 \times 10^6$	169	1.93	69.1	77.7
Chaetoceraceae	<i>Cyclotella cryptica</i>	1323.1	376.3	0.012	0.0034	$3.52 \times 10^6$	610	3.52	218.9	60.8
	<i>Chaetoceros debilis</i>	188.1	165.1	0.002	0.0019	$2.81 \times 10^6$	187	1.14	81.5	83.6
Cymbellaceae	<i>Bacillaria paxillifer</i>	107	45.1	0.001	0.0006	$2.34 \times 10^6$	720	2.37	72.4	69.9
	<i>Phaeodactylum tricornutum</i>	886.4	178.5	0.013	0.0027	$2.15 \times 10^6$	80	4.85	153.9	12.4

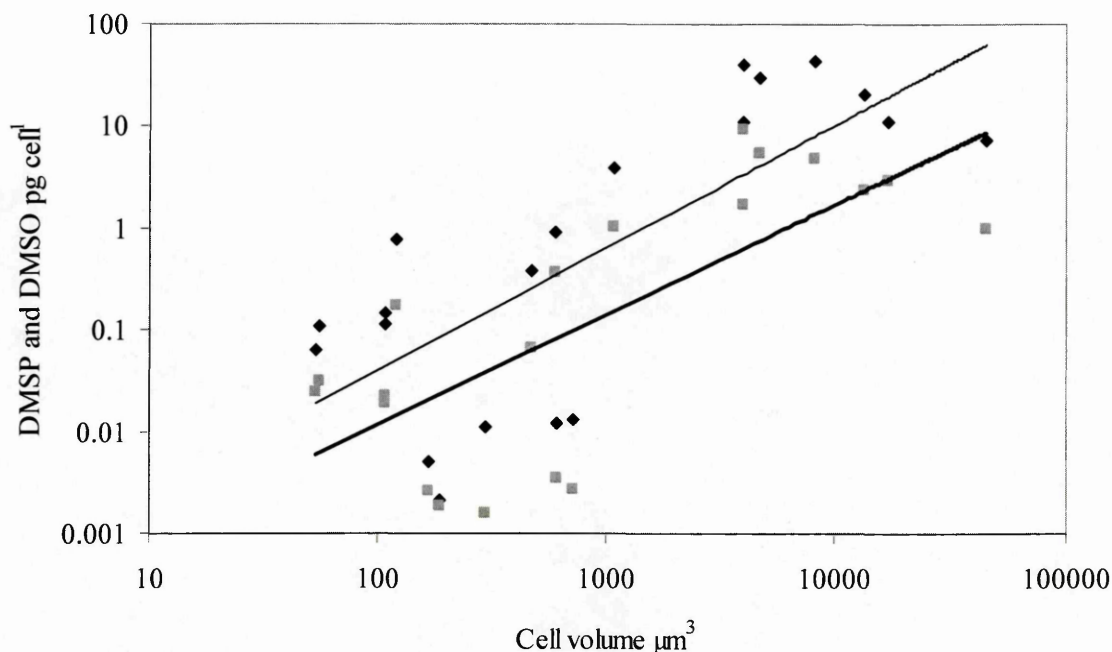
Intracellular DMSPp and DMSOp concentrations were highly correlated ( $r^2=0.926$ ,  $p<0.001$ ) (Fig. 3.5). The DMSPp:DMSOp ratios vary from 1.14 (*Chaetoceros debilis*) to 9.32 (*Skeletonema costatum*) with an average ratio for all cultures of 4.85 (Table 3.3). Overall, DMSOp constitutes between 7-47 % of the particulate methylated sulphur pool (DMSPp and DMSOp). Whilst there is a significant correlation between these compounds (Fig. 3.5) and the algal taxa (Fig. 3.4), a one-Way ANOVA showed no significant relationship between the DMSPp:DMSOp ratio and taxonomic class ( $F_{2,21}=2.80$ ;  $P=0.087$ ). Therefore, whilst individually DMSOp and DMSPp are strongly correlated to taxonomic grouping, the relative proportion of these compounds does not appear to be a function of phylogeny.



**Figure 3.5** Relationship between cellular DMSP and DMSO plotted on a log scale. A positive correlation was observed ( $r^2=0.926$ ,  $p<0.001$ ).

Despite the strong correlation between DMSOp and algal taxa, cellular concentrations of DMSOp vary over 1-2 orders of magnitude within the taxonomic groups. Even within genera, DMSOp concentrations vary considerably within individual species. For example, *Amphidinium operculatum* has 9.39 pg DMSOp cell<sup>-1</sup>, whilst *Amphidinium carterae* has 1.03 pg DMSOp cell<sup>-1</sup>. The DMSPp concentrations between *Amphidinium* species also vary by an order of magnitude with *A. operculatum* having 40.1 pg DMSPp cell<sup>-1</sup> and *A. carterae* with 3.84 pg DMSPp cell<sup>-1</sup>.

The cell volume was calculated for all phytoplankton species analysed for DMSPp and DMSOp. Larger cells were associated with higher DMSPp and DMSOp concentrations than smaller cells (Fig. 3.6). However there was no significant relationship between DMSOp and DMSPp production and cell volume (DMSPp:  $F_{20}=1.18$ ;  $P=0.290$ ; DMSOp:  $F_{20}=0.7$ ;  $P=0.412$ ) (Fig. 3.4). A one way analysis of co-variance (ANCOVA) was used to determine whether size had an effect on taxonomic grouping and the DMSPp and DMSOp concentration (DMSPp: Size:  $F_{1,21}=63.4$ ;  $p=0.457$ ; Taxa:  $F_{2,21}=819.0$ ;  $p=0.004$ ; DMSOp: Size:  $F_{1,21}=3.5$ ;  $p=0.336$ ; Taxa:  $F_{2,21}=25.2$ ;  $p=0.006$ ). Size was shown to have no significant effect as a co-variant factor, whilst taxonomic grouping had a high significant effect.

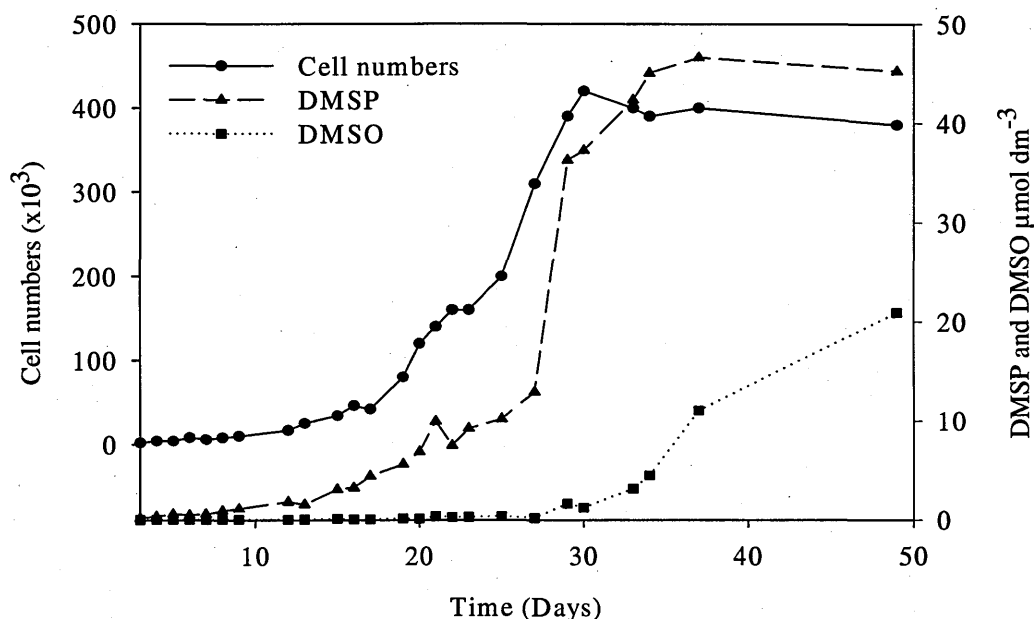


**Figure 3.6** Relationship between algal cell volume and cellular DMSPP and DMSOp concentration plotted on a log scale. There is no significant relationship between cell size and either DMSPP ( $\diamond$ ) ( $F_{20}=1.18$ ;  $P=0.290$ ) or DMSOp ( $\square$ ) ( $F_{20}=0.7$ ;  $P=0.412$ ).

### 3.3.2 Effect of growth on DMSPP:DMSOp values

To monitor the influence of cell growth on the DMSPP:DMSOp ratio, the concentration of DMS, DMSOp, DMSPP, and cell numbers were measured throughout the growth curve of the dinoflagellate *Amphidinium carterae* (Fig. 3.7). DMSOp was detected throughout the experiment and increased exponentially with cell number and DMSPP, with average log-phase intracellular DMSPP and DMSOp concentrations of  $2.70 \pm 0.9$  and  $0.12 \pm 0.09$  pg cell<sup>-1</sup> respectively. The average DMSPP:DMSOp ratio was calculated to be  $26.0 \pm 17.8$  over the experiment. However, whilst the concentration of intracellular DMSPP closely followed that of cell growth, intracellular DMSOp showed a strong increase in concentration at stationary phase, indicating that the relative proportion of the

two compounds varies over the growth cycle. Results showed an average DMSPp:DMSOp ratio in early logarithmic growth of  $11.33 \pm 3.52$ , increasing to an average of  $31.78 \pm 16.63$  over full log-phase and decreasing to  $7.7 \pm 5.25$  during stationary phase. In the dissolved phase, DMSOd concentrations dominated throughout the growth curve, with the highest concentrations observed during stationary phase.

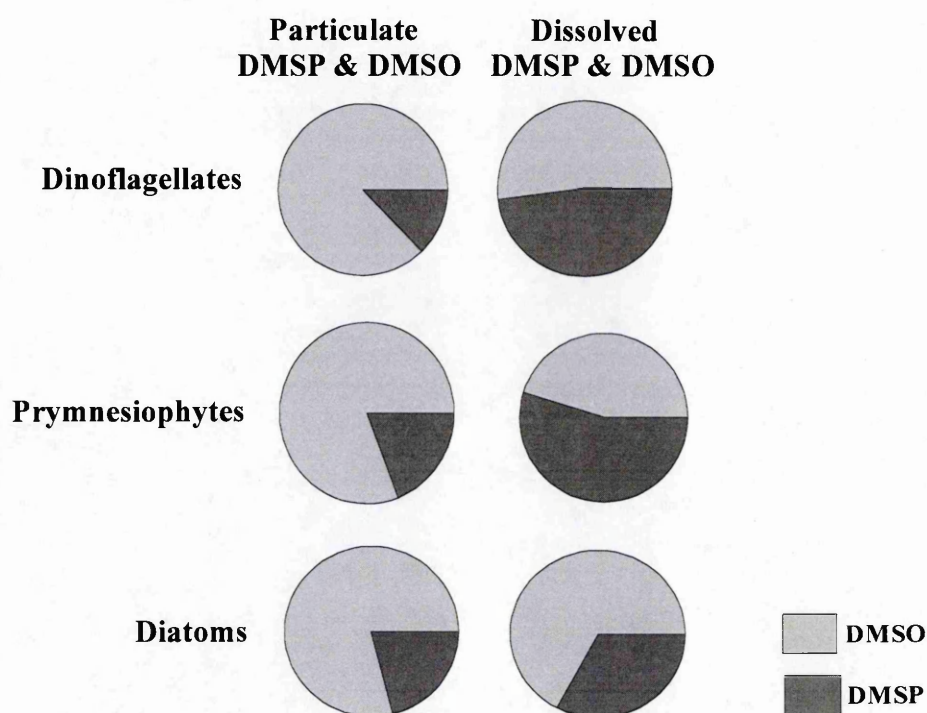


**Figure 3.7** Growth in culture of *Amphidinium carterae* and associated changes in particulate DMSPp ( $\blacktriangle$ ) and DMSOp ( $\blacksquare$ ) concentrations

### 3.3.3 The dissolved fraction of DMSO

During the analysis of DMSPp and DMSOp associated with the algal cultures, dissolved concentrations of DMSPd and DMSOd were measured from the filtrate (Table 3.3). The highest concentrations of DMSOd were associated with the dinoflagellates cultures, ranging from 12 to 353  $\text{nmol dm}^{-3}$  with an average of 131  $\text{nmol dm}^{-3}$ . Concentrations of DMSOd in prymnesiophyte cultures ranged from 4-72  $\text{nmol dm}^{-3}$ , with an average

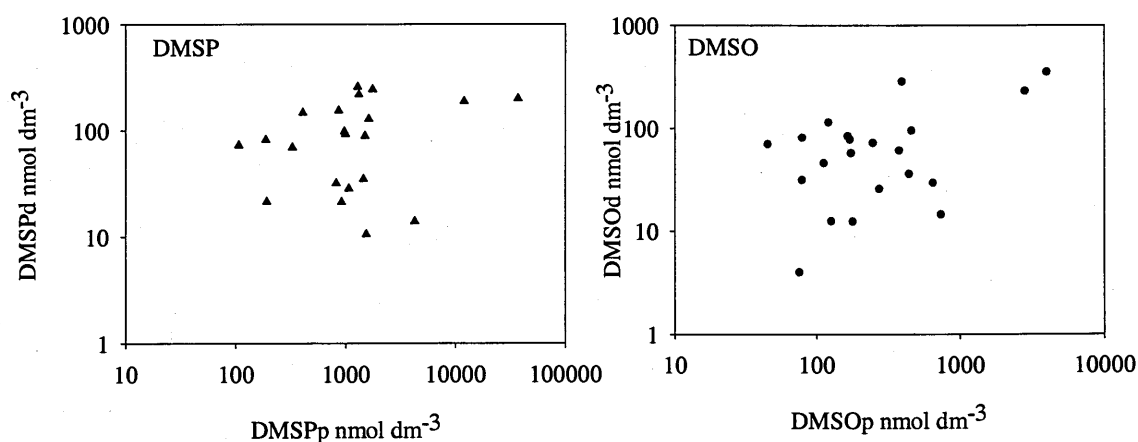
concentration of  $45 \text{ nmol dm}^{-3}$ . In diatom cultures, DMSOd concentrations ranged from  $12\text{--}84 \text{ nmol dm}^{-3}$  with average concentration of  $56 \text{ nmol dm}^{-3}$ . Comparison of DMSOd concentrations between the phylogenetic groups should be considered carefully and in the context of cell densities, which are considerably higher for the diatom cultures (Table 3.3). The DMSPd and DMSOd values as reported in Table 3.3 ( $\text{nmol dm}^{-3}$ ) are shown in comparison with DMSPp and DMSOp, also calculated to  $\text{nmol dm}^{-3}$  (Fig 3.8).



**Figure 3.8** Levels of DMSOd and DMSPd associated with different algal groups in comparison with particulate concentrations of DMSO and DMSP.

Figure 3.8 highlights that while DMSPp exceeds DMSOp on average by 5 times (Section 3.3.1), DMSOd concentrations of  $4\text{--}284 \text{ nmol DMSO dm}^{-3}$  are more similar to DMSPd concentrations which range from  $10.5\text{--}257 \text{ nmol DMSO dm}^{-3}$ . In addition, for one taxonomic group, the Prymnesiophytes, overall DMSOd concentrations exceed those of

DMSPd. An average for all three taxonomic groups shows 15 % of total DMSO (DMSOp+DMSOd) is found in the dissolved form, compared to 7 % for DMSP (DMSPp+DMSPd). Furthermore, whilst there is no significant relationship between DMSPp and DMSPd (DMSP:  $r^2=0.38$ ,  $p=0.089$ ), a significant correlation is observed between DMSOp and DMSOd (DMSO:  $r^2=0.505$ ,  $p=0.019$ ) (Fig. 3.9).



**Figure 3.9** Relationship between the dissolved and particulate fractions associated with algal cultures for DMSP and DMSO.

### 3.4 Discussion

#### 3.4.1 DMSO and Taxonomy

This study demonstrates that phytoplankton species from three major algal taxonomic groups possess the ability to synthesise DMSOp. Furthermore, it was shown that in common with DMSPp (Keller et al., 1989), the dinoflagellates and prymnesiophytes were the major-producers, with diatoms being relatively minor DMSOp producers. The correlation between cellular DMSPp and DMSOp was highly significant across all taxonomic groups with an average intracellular DMSPp:DMSOp ratio of  $4.85 \pm 2.75$  in early log-phase growth. These results indicate that the production of DMSOp by marine phytoplankton maintained in laboratory cultures is both taxon-specific and closely related to DMSPp production.

The significant correlation between DMSOp and DMSPp for laboratory cultures of phytoplankton supports previous field-based measurements. In Scottish coastal waters, a significant correlation was observed throughout the year, with an average DMSP:DMSO ratio of  $10.64 \pm 5.01$  (Hatton and Wilson, 2006). In the Peruvian upwelling region of the Equatorial Pacific, a strong positive correlation between DMSPp and DMSOp was measured, with an average DMSPp:DMSOp ratio of 1.77 (Riseman and DiTullio, 2004). A comprehensive analysis of DMSOp and DMSPp from numerous marine locations, revealed a significant correlation between DMSOp and DMSPp (Simó and Vila-Costa, 2006) with an overall average DMSPp:DMSOp ratio of 4.5. Considering the data from this study and Simó and Vila-Costa (2006) together, it is suggested that DMSOp can



constitute on average 10-20 % of the algal intracellular sulphur pool (DMSP+DMSO) and the concentration of DMSPp is 5 times as high as that of DMSOp.

It should also be noted that although there is high similarity between the DMSPp:DMSOp ratio of 4.85 and 4.5 recorded in this study and oceanic samples respectively, individual algal species have unique DMSPp and DMSOp concentrations. For example, two species of centric diatoms *Skeletonema costatum* and *Thalassiosira pseudonana* have varying ratios of 7.32 and 1.93 respectively. Within the marine environment, Simó and Vila-Costa (2006) noted that a number of environmental factors influence DMSPp:DMSOp ratios including latitude and season. In particular, colder waters *e.g.* coastal Antarctic (Simó and Vila-Costa, 2006) and the Arctic Ocean (Lee et al., 1999; Bouillon et al., 2002) are relatively rich in DMSOp. It is not clear at the moment what causes the elevated DMSOp levels in colder temperatures and possible factors include the algal community composition or algae physiology. One possibility is the cryoprotection effect discussed previously (Section 3.1) and reviewed by Lee et al. (2001).

The benchmark paper by Keller (1989) on DMSPp concentrations in algae with subsequent correlation to taxonomy has provided an explanation for the observed relationship between DMSPp and phytoplankton assemblages. The identification that DMSOp production is similarly taxonomic dependent should also prove to be useful in the analysis of field data. However, it should be noted that the reason for the relationship between taxonomy and DMSPp/DMSOp still remains unclear. It is possible that size of the algal cell plays a role as the exceptionally small dinoflagellate *Gymnodinium simplex*

( $108 \mu\text{m}^3$ ), had DMSPp and DMSOp values similar to prymnesiophytes (Table 3.3).

However, in general the size of algae cells did not have a significant effect on either DMSP or DMSO concentration. Alternative explanations may relate to cell physiology as the diatoms may have considerably lower structural sulphur requirements and may be better adapted to nitrogen uptake (Matrai and Keller, 1994). Dinoflagellates may simply be better storing sulphur in excess of basal sulphur requirements (Chau et al., 1967).

Alternatively the varying concentrations of DMSPp and DMSOp may be related to their 'secondary functions' *e.g.* cryoprotection, stabilisation of cellular proteins or anti-oxidation (Yancey, 2005) (discussed in Section 3.4.2). An alternative explanation could relate to the evolution of the different phytoplankton classes. Previous work has identified that elemental composition of phytoplankton reflects the cells genetic heritage (Quigg et al., 2003) and this may be also be the case for DMSPp and DMSOp.

### 3.4.2 Effect of growth on DMSP:DMSO ratio

Although the DMSPp:DMSOp ratio appears to be consistent within a wide range of phytoplankton species in early log-phase growth (Table 3.3), analysis of *Amphidinium carterae* indicates that the ratio of DMSPp to DMSOp can change significantly over the course of algal growth (Fig. 3.7). The data clearly shows that during log-phase growth, DMSOp constituted only a small percentage (3 %) of the intracellular dimethylated sulphur pool, however this changed significantly in stationary phase where DMSO constituted between 6-30 % of this intracellular pool. These results confirm the finding of Simó et al., (1998), who showed a mean DMSPp:DMSOp molar ratio of *ca.* 25 for log-phase growth in *A. carterae*, that decreased to a minimum of approximately 3 during

the stationary phase. These results highlight the importance of stressing the fact that the data in Table 3.3 reflects values for algal cells in early exponential phase.

The growth experiment should be repeated to validate these findings using different algal species. Future experiments should consider monitoring the algal cultures for bacterial abundance and also being able to discriminate between live and dead cells when the culture enters the lag phase of the growth curve. In addition, consideration should be given to portraying the data on a per cell basis. For example, a closer look at the DMSP and DMSO concentrations on a per cell basis for *A. carterae* suggests that both DMSP and DMSO decrease on a per cell basis until day 29 of the experiment. After Day 29, when cell concentrations decline, both DMSP and DMSO per cell increase for the remainder of the experiment. Because dissolved concentrations of DMSP and DMSO and other ancillary data *e.g.* pigments and cellular carbon, were not measured during this experiment, no further representation of the dataset was included in the results section of this Chapter. Instead, it is possible to speculate on the possible reasons behind these findings. The increase in DMSOp may reflect the cells need to dissipate excess cellular sulphur (discussed in Section 3.4.3). One possible method to test this hypothesis is to grow the algal cultures in varying concentrations of sulphur relative to nitrogen and investigate the relative quantities of DMSOp released into solution.

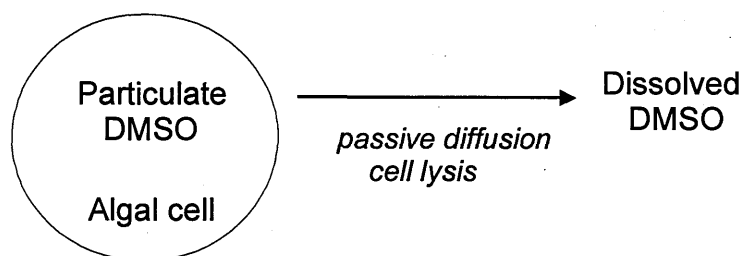
Alternatively the intracellular production of DMSOp could be part of an antioxidant system, whereby the shift in intracellular DMSPp:DMSOp ratio at the stationary phase is an indication of nutrient exhaustion, oxidative stress or due to degradation of proteins. DMSP and associated products (DMS, DMSO, MSNA, acrylate) readily scavenge

hydroxyl radicals and other reactive oxygen species (Sunda et al., 2002). The role of DMSO as a cellular antioxidant is also supported by Risemann and DiTullio (2004) who suggested that DMSPp and DMSOp may play a role as a free radical scavenger based on observations of iron limitation. In addition, Simo and Vila (2006) suggested DMSPp:DMSOp ratio could be a consequence of solar-induced oxidative stress. However, the anti-oxidant hypothesis does not explain why DMSOp would accumulate within the algal cells as observed in this study. The accumulation of DMSOp suggests that it is not solely a transient compound as part of an antioxidant cascade within algal cells.

At the present, additional research on the intracellular role of DMSO is required to resolve any possible cellular anti-oxidant function. To my knowledge, the production of DMSP and DMSO (and associated methylated compounds: DMS, MSNA) in response to oxidative stress has never been measured simultaneously. It should also be noted that an algal response to oxidative stress may be species specific (Rijstenbil, 2003) and therefore a range of species should be tested. Furthermore, other anti-oxidant molecules *e.g.* carotenoids, ascorbate, glutathione (Asada, 1999); and scavenging enzymes *e.g.* superoxide dismutase and ascorbate peroxidase (Niyogi, 1999) may also be involved and under varying stress conditions, alternate anti-oxidants may be used by the algae. It is possible that further insight on algal-DMSO could be gained from some of the biotechnical applications of DMSO, *e.g.* the permeabilization and fusion of cell membranes (Anchordoguy et al., 1992).

### 3.4.3 Dissolved fractions of DMSO and DMSP

The extracellular concentrations of DMSOd recorded in algal cultures (4–285 nmol dm<sup>-3</sup>) are comparable to DMSPd concentrations (10.5–257 nmol DMSO dm<sup>-3</sup>). This contrasts with the intracellular concentrations, where DMSPp typically exceed DMSOp concentrations by ~ 5 times (Table 3.3). To account for the smaller variation in the dissolved values of DMSO and DMSP compared to particulate concentrations, it is likely that higher quantities of DMSOp are released from the cell to the dissolved fraction, compared to DMSPp. In addition, it has been suggested that dissolved concentrations of DMSOd are not turned over by bacteria as quickly as DMSPd or DMS (Simó et al., 2000). The potential presence of bacteria in the algal cultures analysed in this study can not be ruled out. An increased loss of DMSOp from algal cells compared to DMSPp is a logical assumption based on the ability of DMSO to permeate biological membranes (Liu et al., 1997) (Fig. 3.10). Furthermore, an algal cell could benefit from the diffusive loss of DMSOp if it is linked to an overflow mechanism for exuding excess sulphur and as a means of dissipating energy. Cells require a buffering mechanism to regulate cysteine and methionine levels and as such it has been hypothesized that DMSPp may be an overflow mechanism (Stefels, 2000). It is therefore possible that the conversion of DMSPp to DMSOp, via DMS may be an important component within any overflow mechanism.



**Figure 3.10** Diagram showing permeative loss of DMSO from algal cells into solution

The permeative loss of cellular constituents due to diffusive processes is based on the concentration gradient that exists between the external and internal fractions of DMSO and this should be carefully considered. A review on the excretion of metabolites and associated diffusion transport mechanisms can be found in Fenchel et al. (1998). With regards to this study, as an example, the dinoflagellate, *P. micans* (Creran), has a concentration of  $1.72 \text{ pg DMSOp cell}^{-1}$  and a cell volume of  $4000 \text{ } \mu\text{m}^{-3}$  (Table 3.2). The conversion to  $\text{dm}^{-3}$  of cell volume equates to  $13.4 \text{ mmol DMSOp dm}^{-3}$  of cell volume. This value is 6 orders of magnitude greater than the external dissolved concentration of  $12.5 \text{ nmol DMSOd dm}^{-3}$ . In comparison, the intracellular:extracellular ratio for DMSP in *P. micans* (Creran) is  $2.7 \times 10^6$ . However, *P. micans* has one of the largest concentration differences observed, and an overall intracellular:extracellular ratio for dinoflagellate DMSP and DMSO is  $1.28 \times 10^6$  and  $0.35 \times 10^6$  respectively. Prymnesiophytes have an overall average intracellular:extracellular ratio of  $0.32 \times 10^6$  and  $0.96 \times 10^6$  for DMSP and DMSO respectively. Diatoms have smaller concentration differences and overall average intracellular:extracellular ratios for DMSP and DMSO are  $0.011 \times 10^6$  and  $0.017 \times 10^6$  respectively. The intracellular:extracellular ratios highlight the potential for diffusive loss of DMSO from algal cells belonging to these phylogenetic groups. The

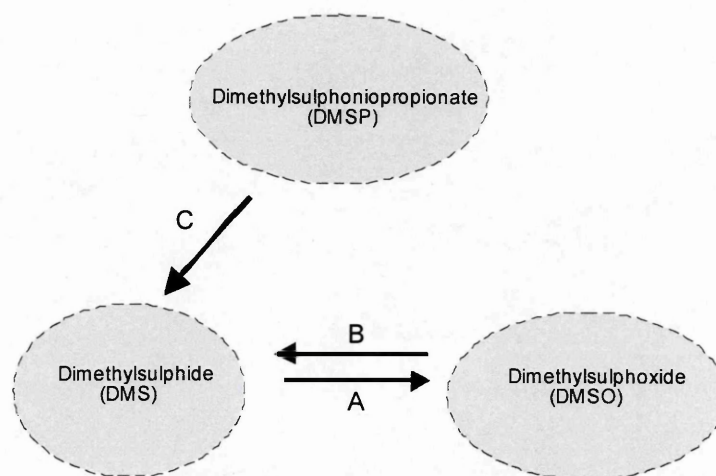
ratios also suggest that there is continual production of DMSO within algal cells and support the concept that DMSO is an important component of cellular methylated sulphur. It was recently observed by Vila-Costa et al.(2006a) that certain phytoplankton groups can incorporate  $^{35}\text{S}$ -DMSP. This was demonstrated for non-DMSP producers such as the cyanobacteria, and also for diatom cultures of *Thalassiosira pseudonana* and *T. oceanica*. It is not currently understood what cellular mechanism is used by phytoplankton cells to incorporate the DMSP. In addition, it is not evident whether phytoplankton can also incorporate DMSO and whether this is more common than DMSP.

It is also noteworthy to consider the analysis of DMSOd. The analytical procedures used to measure DMSPd have recently been evaluated raising the issue of wide-spread over-estimation of the DMSPd pool (Kiene and Slezak, 2006). The methodological issues highlighted by Kiene and Slezak (2006) predominantly dealt with gravity filtration through Whatmann glass fibre filters, and not the syringe filtration employed by this study. However any analysis of dissolved fraction should always consider potential artificial lysis of cells, which will increase the dissolved pool. In this study, attention was made to keep the sample volumes to a minimum (typically between 3-5 cm<sup>3</sup> was filtered) and the filtration pressure was always gentle.

#### **3.4.4 The role of DMSO released from algal cells in the wider marine sulphur cycle**

The role of DMSO in methylated sulphur cycling *i.e.* interactions between DMSP and DMS (Fig. 1.5) are not currently well understood. Recent studies have focused on

identifying the micro-organisms involved and quantifying the transformations of the different sulphur compounds (Fig. 3.11).



**Figure 3.11** The interaction between DMSP, DMS and DMSO based on our current knowledge. The organisms currently identified that carry out the transformations highlighted in Fig. 3.11 are listed below: **A:** *Methylobacterium* (Fuse et al., 1998); *Methylophaga* (Vila-Costa et al., 2006b); *Roseobacter* (Gonzalez et al., 1999). **B:** *Roseobacter* (Gonzalez et al., 1999); *Proteobacter* (Visscher and van Gemerden, 1991a); *Methylophaga* (Vila-Costa et al., 2006b) **C:** *Roseobacter* (Gonzalez et al., 1999), *Marinomonas* (Dodd et al., 2006).

The connection between DMSO and DMS was highlighted during incubations of seawater samples with the addition of different substrates (Vila-Costa et al., 2006b). A high rate of conversion of DMSO to DMS was observed when DMSO was added. Conversely, DMS was also transformed to DMSO yet this process required in the presence of high levels of added glucose or methanol, or only endogenous substrates (Vila-Costa et al., 2006b). More recently, del Valle et al. (2007) analysed the transformation of DMS using  $^{35}\text{S}$ -DMS. DMSO was identified as the main sulphur product (~72 %) resulting from the metabolism of DMS in the surface mixed layer. The microbiological production rates of DMSO ranged from 0.07 to 0.33 nmol dm<sup>-3</sup> day<sup>-1</sup>, which represented a turnover time for DMSO of 15-61 days (del Valle et al., 2007).



This Chapter highlights that the release of DMSO from algal cells can also contribute to the ambient pool of DMSOd. It is not apparent at present how the rates of DMSO release from algae compare to the microbial transformation rates of DMS to DMSO in the marine environment. It is possible that as suggested by del Valle et al. (2007), the rates of DMSO production by microbial transformations are exceeded by the release of DMSOd from algal cells.

### 3.5 Conclusions

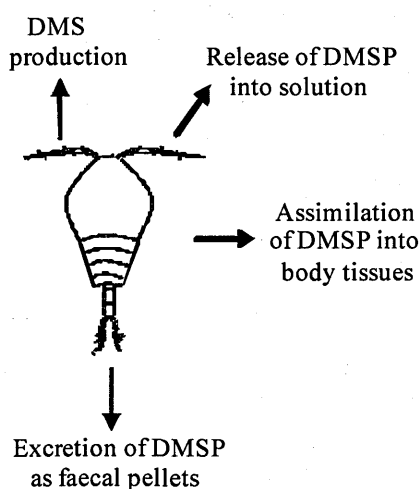
DMSOp concentrations are shown to be highly species-specific for a range of dinoflagellates, prymnesiophytes and diatoms species, and significantly correlated with phytoplankton taxonomic class. An overall average DMSPp:DMSOp ratio was calculated at 4.85. This ratio, obtained from laboratory phytoplankton cultures, compliments previously reported value of DMSPp:DMSOp ratio of 4.5 for a range of oceanic samples. The strong correlation between algal-associated DMSPp and DMSOp, concentrations indicates that the intracellular function(s) of these compounds is linked. These results are further evidence that DMSOp constitutes a consistent proportion of algal cellular methylated sulphur and should be considered as part of DMSP and DMS cycling in the marine environment.



## Chapter 4. Trophic transfer of DMSP & DMSO

### 4.1 Introduction

Within the marine pelagic environment, zooplankton grazing on phytoplankton is considered to play an important role in the biogeochemical cycling of DMSP, DMSO and DMS. Grazing can induce the release of DMSPp into the surrounding environment and facilitate DMS production. Furthermore DMSPp can be assimilated by grazers and either be retained within their body tissues or subsequently excreted and exported as copepod faecal pellets out of the euphotic zone to deeper waters. Therefore the effects of grazing can be conveniently separated into four categories which are examined in further detail: assimilation of DMSP; release of DMSP into solution; DMS production; and excretion of DMSP as faecal pellets (Fig. 4.1). A list of previous research on this topic where the prey and predator organisms have been identified is provided in Table 4.1. The grazers have been sub-divided into microzooplankton (20-200  $\mu\text{m}$ ) *e.g.* ciliates and heterotrophic dinoflagellates, and mesozooplankton (0.2 – 20 mm) *e.g.* copepods (Sieburth et al., 1978).



**Figure 4.1** Schematic diagram highlighting the different sinks for algal-produced DMSP during grazing.

**Table 4.1** Examples of previous grazing experiments where the identification of both the predator and prey organisms is known. The predator organisms have been divided into micro-grazer (20-200  $\mu\text{m}$ ) and meso-grazer (0.2-20 mm) categories.

Phytoplankton	Micro-grazer (20-200 $\mu\text{m}$ )	Meso-grazer (0.2-20 mm)	Reference
<i>Isochrysis galbana</i>	<i>Strombidium sulcatum</i>	-	Christaki et al. (1996)
<i>Emiliania huxleyi</i>	<i>Oxyhris marina</i>	-	Wolfe et al. (1994)
<i>Isochrysis galbana</i>	<i>Oxyhris marina</i>	-	Archer et al. (2001)
<i>Emiliania huxleyi</i>	<i>Amphidinium longum</i> ; <i>Gymnodinium</i> sp.; <i>Oxyrrhis</i> <i>marina</i> ; <i>Coxiella</i> sp.	-	Strom et al. (2003)
<i>Gymnodinium nelsoni</i> ; <i>Prorocentrum micans</i>	-	<i>Centropages hamatus</i> ; <i>Labidocera aestiva</i>	Dacey and Wakeham (1986)
<i>Phaeodactylum tricornutum</i> ; <i>Thalassiosira weissflogii</i>	-	<i>Eurytemora affinis</i>	Kwint (1996)
<i>Tetraselmis impellucida</i> ; <i>Dunaliella tertiolecta</i>	-	<i>Temora longicornis</i>	Tang et al. (1999)
<i>Tetraselmis impellucida</i>	-	<i>Acartia tonsa</i>	Tang et al. (2000)
<i>Emiliania huxleyi</i> ; <i>Skeletonema</i> <i>costatum</i>	-	<i>Calanus finmarchicus</i>	Levasseur et al. (1996)
<i>Phaeocystis globosa</i> ; <i>Isochrysis galbana</i>	<i>Gyrodinium dominans</i>	<i>Acartia tonsa</i>	Tang and Simo (2003)

#### 4.1.1 Assimilation of DMSP into copepod body tissues

In the pelagic environment there are increasing reports on the accumulation of DMSPp within the body tissues of higher trophic organisms including copepods, euphausiids, fish and shellfish (Iida and Tokunaga, 1986; Tang et al., 1999; Hill and Dacey, 2006). The assimilation of DMSP reflects a role for copepods as components of the 'traditional' food chain; through the grazing of primary producers and in turn representing a food source for higher trophic levels (Cushing, 1989). The assimilation of DMSPp in the digestive tracts and body tissues of copepods has previously been demonstrated for copepods maintained in the laboratory and also for individuals collected from the marine environment (Kwint et al., 1996; Tang et al., 1999; Tang et al., 2000a). Laboratory-based grazing studies revealed DMSPp concentrations in the body tissues of *Temora longicornis* after grazing on *Tetraselmis impellucida* ranged from 0.5 to 3.8 nmol DMSPp copepod<sup>-1</sup> (Tang et al., 1999). During a similar laboratory experiment, but with different organisms: *Eurytemora affinis* grazing on *Phaeodactylum tricornutum*, values of 0.16 nmol DMSPp copepod<sup>-1</sup> were recorded (Kwint et al., 1996). In comparison, *T. longicornis* copepods collected from the field displayed DMSP concentrations ranging from 0.02 to 1.03 nmol copepod<sup>-1</sup> (Tang et al., 1999). The explanation for the accumulation of DMSPp within copepods has been attributed to osmoregulation as it has been demonstrated that certain copepods are capable of adjusting body concentrations of DMSPp to external salinity concentrations (Tang et al., 1999; Tang et al., 2000a; Tang et al., 2000b). Tang et al. (1999) identified that increasing salinity was concomitant with increasing DMSP in *T. longicornis* body tissues reaching 3.8 nmol DMSPp copepod<sup>-1</sup> at a salinity of 30. This suggests that as previously discussed for phytoplankton in Chapter 3, DMSPp can have a role in the osmoregulation of higher trophic organisms.

In addition to the accumulation of DMSP in mesograzers, DMSPp assimilation has also been demonstrated for microzooplankton. In a laboratory experiment, concentration of DMSP within the heterotrophic dinoflagellate *Gyrodinium dominans* on *Isochrysis galbana* and *Phaeocystis globosa*, was 0.01 and 0.1 nmol DMSP ind.<sup>-1</sup> respectively (Tang and Simo, 2003). These concentrations represented the retention of 32 % and 44 % respectively of the total DMSPp that was ingested by *G. dominans*. Other researchers have based estimates of DMSPp assimilation by microzooplankton on the fraction of DMSPp lost that was not accounted for by DMSPd and DMS production. For example, Christaki et al. (1990) estimated the ciliate *Strombidium sulcatum* released >65 % of cellular-DMSP during grazing on *I. galbana* and assumed the remaining 35 % was incorporated by either *S. sulcatum* or associated bacteria. Similarly, Wolfe et al. (1994) recovered only a fraction of the DMSPp lost as DMSPd or DMS during grazing by *O. marina* on *E. huxleyi* and estimated that consumption by *O. marina* accounted for 25-76 %, depending on cell density of grazers. The authors also speculated on the use of DMSP by the heterotrophic dinoflagellate, suggesting that it may be employed as a methyl donor.

#### 4.1.2 Release of algal-DMSP into solution.

In addition to the assimilation of algal compounds, grazers are also an important source of dissolved organic matter (DOM) which helps to sustain the microbial food web (Nagata, 2000). The importance of grazing-generated DOM to the microbial food web is highlighted by comparing the bacterial DOC demand, which can represent 20-40 % of daily carbon fixation, with the quantity of DOC released by algal cells, which is estimated to be <10 % of daily carbon fixation (Jumars et al., 1989; Strom et al., 1997). The production of DOM by grazing is thought to reconcile the difference and this hypothesis is

supported by results from laboratory experiments. For example, the direct DOC release from algal exudates represented 3-7 % of algal carbon content and increased to 16-37 % in the presence of *Calanus pacificus* (Strom et al., 1997). Other laboratory grazing experiments using *Acartis tonsa* and the diatom *Ditylum brightwelli* and the dinoflagellate *Ceratium lineatum*, demonstrated that up to 50-60 % of grazed carbon can be released into solution (Møller and Nielsen, 2001). The release of this DOM occurs through the leakage of DOM from the mouthparts of zooplankton, also known as 'sloppy feeding' (Conover, 1966) and the excretion and subsequent dissolution of faecal pellets (Section 4.1.4). One of the parameters controlling the release of DOC during grazing is the predator-prey size ratio, whereby predation on proportionally larger prey can release more carbon to the ambient environment than grazing on smaller prey (Møller, 2005).

Similar to other algal constituents, DMSPp is also released into solution during grazing and thereby contribute to the extracellular pool of DMSPd. An increase in the levels of DMSPd was observed during grazing by *Oxyhris marina* on *Emiliana huxleyi*, (Wolfe et al., 1994). An increase in DMSPd of  $0.5 \text{ nmol dm}^{-3} \text{ hr}^{-1}$  was also reported during grazing experiments with *Eurytemora affinis* on *Phaeodactylum tricornutum* (Kwint et al., 1996). However, an increase in DMSPd concentrations in the presence of grazers is not always observed. Concentrations of DMSPd did not change when copepods were added to incubations of natural seawater samples in the Mediterranean Sea (Christaki et al., 1996). Similarly, levels of DMSPd were not affected by the addition of *Calanus finmarchicus* to mesocosms containing *Emiliana huxleyi* blooms (Levasseur et al., 1996). The absence of any increase in DMSPd concentrations in the presence of grazers, or when algal blooms are crashing has been attributed to high prevailing bacterial activity as DMSPd is rapidly metabolised by bacteria (Levasseur et al., 1996). Natural assemblages of bacterioplankton

appear to rapidly metabolise DMSPd which can supply 1-15% of the carbon and virtually all of the sulphur demand of the bacterioplankton (Kiene and Linn, 2000; Simo et al., 2002).

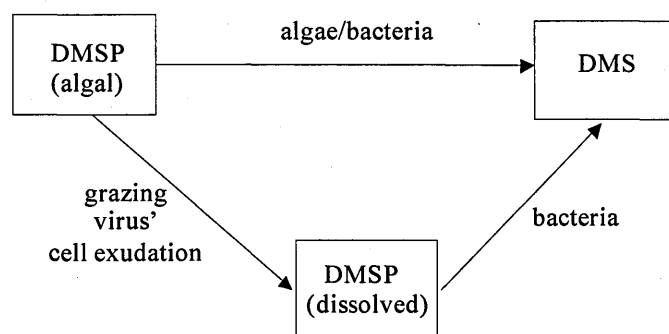
#### 4.1.3 DMS production

One of the earliest observations on the effects of grazing on DMSP biogeochemistry was an increase in DMS concentrations of  $200 \text{ nmol dm}^{-3} \text{ day}^{-1}$  during grazing by the copepods *Centropages hamatus* and *Labidocera aestiva* on the dinoflagellate *Gymnodinium nelsoni* (Dacey and Wakeham, 1986). Subsequently, the link between grazing and DMS concentrations has been corroborated in numerous laboratory, mesocosm and field experiments. For example, during experiments in the Southern Ocean, the addition of krill to natural assemblages of seawater resulted in a 3 to 16-fold increase in DMS concentrations (Daly and DiTullio, 1996). More recently, DMS production rates in the North Sea of  $0.46\text{--}22.9 \text{ nmol dm}^{-3} \text{ day}^{-1}$  were correlated with microzooplankton grazing rates (Archer et al., 2003). However, a correlation between DMS and grazing has not always been observed, and previous work showed DMS concentrations remained constant at  $\sim 1 \text{ nmol dm}^{-3}$  during a 4 hr grazing experiment involving the algae *Phaeodactylum tricornutum* and the copepod *Eurytemora affinis* (Kwint et al., 1996).

The variation observed in the production of DMS due to grazing may result from the way in which it is produced. There are currently two major possibilities for DMS production (Fig. 4.2): the first is that DMS may be released directly from the algal cell due to grazing-induced contact between DMSP and the DMSP-lyase enzyme (Christaki et al., 1996; Wolfe and Steinke, 1996; Archer et al., 2001). It has also been hypothesized that DMSP may act as a grazing deterrent in algae, whereby the resulting products, DMS and acrylate,



deter grazers (Wolfe and Steinke, 1996; Wolfe, 2000). This would suggest that DMSP cleavage to DMS is initiated by the algal cells. There appears to be some support for this hypothesis as high DMSP-lyase activity in *Emiliania huxleyi* during laboratory experiments was associated with lower grazing rates (Strom et al., 2003). For a full review on the role of dimethylsulphide and other volatile compounds as feeding chemical cues see Steinke et al. (2002).



**Figure 4.2** Schematic diagram highlighting different pathways by which DMS can be produced from DMSP

The alternative pathway for DMS production is via the release of DMSP from algal cells into solution where it is subsequently degraded to DMS by bacterioplankton. The microbial production of DMS from DMSPd has been well documented, both in this thesis (Section 1.1.4) and in the wider literature (Kiene and Service, 1991; Taylor and Gilchrist, 1991; Yoch, 2002). The importance of bacteria in DMS production is further highlighted by the microbial control hypothesis which considers DMS production as a net result of bacterial sulphur demand relative to available DMSP (Kiene et al., 2000). The link between grazers, prey and bacteria was highlighted by Wolfe et al. (1994) who found an increase in DMS concentrations was associated with an increase in bacterial abundance during grazing by *Oxyrrhis marina* on *Emiliania huxleyi*. Furthermore despite the wide

variations in the loss of DMSPp ( $3\text{--}38 \text{ nmol dm}^{-3} \text{ d}^{-1}$ ), DMS values were relatively constrained ( $0.7\text{--}3.4 \text{ nmol dm}^{-3} \text{ d}^{-1}$ ), which the authors suggested was evidence of a bacterially-mediated decoupling step.

Resolving the pathway of DMS production in the marine environment (Fig. 4.2) and the organisms responsible *in situ* production is currently a major challenge in research on DMS biogeochemistry. Previous efforts focused on the identification of the DMSP-lyase enzyme within bacterioplankton and algae. However until it is possible to distinguish between the DMSP-lyases of bacteria and algae, there is no apparent way of identifying the relative proportion of each pathway (Yoch, 2002). More recently, a new pathway for DMS production was identified (Fig. 1.4) (Dodd et al., 2006), although its relative importance in the wider marine environment is currently unclear.

#### 4.1.4 Excretion.

The excretion of faecal pellets by marine copepods has previously been described as a ‘small-scale process with large scale implications’ (Kjørboe, 2001) due to its role in the sequestration of anthropogenic carbon dioxide. Copepod faecal pellets are an important mechanism by which organic matter is transported from the upper ocean to the ocean floor (Longhurst, 1991). The production, composition and fate of copepod faecal pellets in the marine environment is comprehensively reviewed by Turner (2002) and Frangoulis et al. (2004). One of the pertinent issues emerging from these reviews is that the downwards flux of organic matter is often dominated by the faecal material of macrozooplankton and fish. The smaller faecal pellets of micro-zooplankton and meso-zooplankton are mostly recycled within the water column. This highlights the faecal material as an important sites

for nutrient decomposition and recycling within the water column (Alldredge and Silver, 1988; Simon et al., 2002). The rapid turnover of faecal pellets is reflected in the bacterial abundance and productive associated with the marine particles that are orders of magnitude higher than in the ambient water (Caron et al., 1982; Grossart et al., 2003). Tang et al. (2001) estimate that 1 cm<sup>3</sup> of copepod faecal material contained about 10<sup>10</sup> to 10<sup>11</sup> DMSP-consuming bacteria. A review on the microbial ecology of organic aggregates in the aquatic environment is provided by Simon et al. (2002), though the review encompasses all particulate material and aggregates in the pelagic environment. In comparison to general particulate material, faecal pellets are likely to have a higher density and surrounded by a peritrophic membrane (Ferrante and Parker, 1977; Yoshikoshi and Ko, 1988). The size of mesozooplankton faecal pellets range from ~50 µm to a few hundred µm in length and in turn the size of faecal material is influenced by the size of organism; ingestion rates and food concentrations, as reviewed by Frangoulis et al. (2004).

Within regards to DMSP dynamics, the grazing by copepods on DMSP-producing phytoplankton and the subsequent excretion of faecal pellets produces a source of DMSPp within faecal pellets in the upper water column. During a laboratory grazing experiment using *Eurytemora affinis* grazing on *P. tricornutum*, between 10-50 % of ingested DMSP was exported as faecal material (Kwint et al., 1996). A similar experiment, but using different copepods and prey organisms found that during the grazing of *Acartia tonsa* on *Tetraselmis impellucida*, 0.1% of DMSP ingested was exported as faecal material (Tang, 2001). The discrepancy between these two studies was attributed to a difference in faecal material as a result of different copepod species, diets and/or food concentration (Tang, 2001). Within the wider marine environment, krill have also been found to egest a significant quantity of DMSP within their faecal pellets (6.67 nmol ind<sup>-1</sup>) (Daly and

DiTullio, 1996). However, in the Antarctic coastal environment although particle fluxes were dominated by krill faecal pellets, concentrations of DMSPp in sediment trap material were low and the authors concluded that DMSPp was leaching from the faecal pellets into the ambient seawater (Daly and DiTullio, 1996).

#### 4.1.5 Effect of grazing on algal-DMSO

To date, most studies have quantified the effects of grazing on biogenic sulfur through the analysis of DMSP and DMS. DMSO is an additional algal compatible solute that can represent one fifth of algal methylated sulphur (see Chapter 3). During experiments in the North Water Polynya it was demonstrated that in the presence of copepods, DMSOd concentrations increased at a rate of  $0.1\text{--}18.5\text{ nmol DMSO dm}^{-3}\text{ day}^{-1}$  in comparison to the rate of DMSPd increase which ranged from  $0.04\text{--}8.14\text{ nmol dm}^{-3}\text{ day}^{-1}$  (Lee et al., 2003). Increased concentrations of DMSOd were also measured during grazing by *O. marina* on *E. huxleyi*, suggesting that micro-zooplankton grazing can also enhance the concentrations of DMSOd in seawater (Darroch, 2003). Furthermore, as discussed in Chapter 3 (Section 3.4) once DMSO is released into the marine environment, its potential role as an assimilatory carbon and sulphur source and to what extent it is converted to DMS is currently unclear.

It can therefore be seen, that through the selective consumption and processing of their food, zooplankton influence the flux between particulate and dissolved organic matter and the biogeochemical cycling of major nutrients, such as DMSP and DMSO. This study monitors the fate of algal-DMSP and DMSO in the presence of grazers. The calanoid

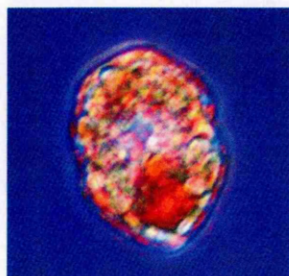
copepods *Acartia clausi* and *Temora longicornis* were used as grazers and the prey was the dinoflagellate *Scrippsiella trochoidea*. Specific objectives were to:

- 1) Measure the sinks of algal-DMSP during grazing
- 2) Investigate species-specific differences by using two species of copepod.
- 3) Investigate bacterial metabolism of dissolved DMSP, DMSO and DMS

## 4.2 Method

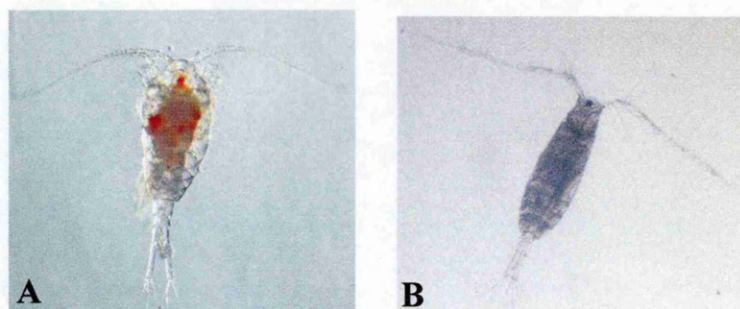
### 4.2.1 Cultures of phytoplankton and copepods

The dinoflagellate *Scrippsiella trochoidea* was used as the algal prey organism in all the grazing experiments in this study (Fig. 4.3). *S. trochoidea* is a ubiquitous coastal dinoflagellate, which was previously identified as a major producer of DMSPp (Keller et al., 1989) and DMSOp (Chapter 3). Stock cultures of *S. trochoidea* were obtained from the Culture Collection of Algae and Protozoans (CCAP) and grown in Kmin media (Leftley et al., 1987) at 14 °C on a 14:10 light:dark cycle. Although certain dinoflagellates are notorious for their unpalatability and toxicity, this was not observed with *S. trochoidea* which has no previous reports of toxicity (Hold et al., 2001). Grazing experiments were also attempted with another dinoflagellate, *Amphidinium operculatum*. However the copepods did not actively graze on this phytoplankton species, as demonstrated by the lack of decline in algal cell numbers and DMSPp or DMSOp for >3 hours. A review of the literature suggested this was due to toxicity and *A. operculatum* was no longer used in grazing experiments (Nayak et al., 1997; Jeong et al., 2001).



**Figure 4.3** Dinoflagellate *Scrippsiella trochoidea* (length 40  $\mu\text{m}$ ).

The grazing experiments used one of two copepods: *Temora longicornis* or *Acartia clausi* (Fig. 4.4). Both species are widely cited in the scientific literature and are common neritic copepods (Mauchline, 1998). *T. longicornis* is slightly larger in size (1.4 mm), whilst *A. clausi* is smaller (1.1 mm). Both species of copepods were isolated from a vertical zooplankton trawl in Loch Linnhe, in July 2004, using a 68 µm mesh-sized zooplankton net. Populations were maintained in culture through successive generations prior to experimental use. Both copepod cultures were maintained on a mixed diet of *Rhinomonas reticulatum* and *Oxyrrhis marina* phytoplankton. During the grazing experiments only adult individuals were used and the same copepod individuals were never used twice to avoid any behavioural effects



**Figure 4.4** a: *Temora longicornis* (length 1.4mm); b: *Acartia clausi* (length 1.1mm)

#### 4.2.2 Grazing experiments

Grazing experiments were conducted in a 2.5 litre container, containing 1 litre of 0.7 µm filtered seawater (14°C and a salinity of 32). The container was shaded to avoid direct light. *S. trochoidea* from the stock culture were gently added to achieve a final concentration of  $2 \times 10^3$  cells cm<sup>-3</sup>. Concentrations of biogenic sulphur in the media were taken as a background control before the addition of copepods. For each grazing

experiment, 200 adult copepod individuals were used. Prior to the grazing experiments, adult copepods were starved for 48 hours to ensure immediate grazing during the experimental period. A 100  $\mu\text{m}$  mesh was used to transfer the adult individuals to the experimental vessel.

After the addition of copepods, samples were collected for DMS, DMSPp, DMSPd, DMSOp, DMSOd, and cell counts through Teflon tubing attached to a 50  $\text{cm}^3$  syringe. The end of the tubing was covered with 200  $\mu\text{m}$  mesh to prevent zooplankton being collected. The samples were collected at Time<sub>zero</sub> and every 35 mins thereafter throughout the grazing period. The limiting factor for the number of sample analysed was the processing time for DMS analysis. DMS analysis was performed immediately by injecting a sample directly into the purge chamber via a AP25 depth filter. The filter was stored for subsequent DMSPp analysis whilst the filtrate, after being purged, provided material for DMSPd analysis (Section 2.1.2). The same procedure was conducted for DMSO analysis using fresh AP25 depth filters (Section 2.1.2). Experiments with both *T. longicornis* and *A. clausi* grazing on *S. trochoidea* were performed in duplicate.

To measure grazing rates, 3  $\text{cm}^3$  of culture were taken for counting at each sampling point, *i.e.* every 30 min, fixed with 1% Lugols and stored for counting at a later date. Counts were performed manually using a Sedgewick-Rafter counting cell and a Zeiss microscope. The method of estimating grazing rates through the direct counting of algal prey cells is a traditional method, as discussed by Frost (1972). It should be noted that this is just one of many methods involving incubation and gut analyses employed to estimate feeding rates and a comparison of different methods can be found in Kiørboe et al. (1985); Peterson et al. (1990); and Båmstedt et al. (2000).



The assimilation of DMSP into copepod body tissues was measured following the procedure of Tang (2000). After three hours of feeding, copepods were transferred to fresh filtered seawater and allowed to empty their gut contents for 3 hours. Five individual copepods were collected by pipette, washed in fresh filtered seawater and added to 10 cm<sup>3</sup> glass crimp top vials containing 3 cm<sup>3</sup> of 10 M NaOH and 7 cm<sup>3</sup> distilled water. The samples were incubated for a minimum of 12 hours prior to analysis. For each grazing experiment this was performed in triplicate. Analysis for DMSP content was performed by gas chromatography following standard procedure, see Section 2.1.2.

Faecal pellets were retrieved from the experimental vessel immediately after the removal of copepods by siphoning the bottom centimetre of seawater onto a 100 µm mesh which was submerged in seawater to minimise physical disruption of faecal pellets. The faecal pellets were subsequently transferred to a sterile glass Petri dish, which contained fresh autoclaved seawater which had been filtered through a 1 µm filter. This was an important step as even small numbers of *S. trochoidea* could easily distort faecal measurements. The faecal pellets were therefore visually inspected using a light stereo-microscope to ensure the absence of any non-faecal material. To analyse for DMSPp, replicate samples of 50 faecal pellets were added to 5 cm<sup>3</sup> glass vials with 1 cm<sup>3</sup> 10 M NaOH and 4 cm<sup>3</sup> distilled water. Analysis was performed for DMSPp only, using the method previously described (Section 2.1.2)

### 4.2.3 Amendments

During the grazing experiments it was consistently observed that the dissolved concentrations of DMSPd and DMSOd decreased towards the end of the experimental period. This was considered to result from microbial degradation of both compounds and was therefore investigated further through the addition of inhibitors during a repeat of the *T. longicornis* and *S. trochoidea* grazing experiment. Previous studies have used a range of inhibitors to investigate the influence of microbial activity on DMSP degradation *e.g.* chloramphenicol (Kiene, 1990), glycine betaine (Kiene and Gerard, 1995), and dimethyldisulphide (Wolfe and Kiene, 1993). The aim of the inhibitors was to inhibit bacterial consumption of DMSP during the 3 hour incubation period, with no adverse effects on the algal cells. Therefore a preliminary study using two inhibitors separately, glycine betaine (Sigma Aldrich, UK), and kanamycin (Sigma Aldrich, UK), was carried out to see if there was any effect on *S. trochoidea*. Glycine betaine is a naturally occurring structural analogue of DMSP that has been previously demonstrated to inhibit the microbial consumption of DMSPd through competitive inhibition when it was added at concentrations of 1-50  $\mu\text{mol dm}^{-3}$  (Kiene and Gerard, 1995). Kanamycin is an antibiotic that affects RNA translation and is often used in short-term microbiological studies in conjunction with other antibiotics.

Three flasks of *S. trochoidea* cultures were established with the same laboratory conditions as described above with a culture volume of 200  $\text{cm}^3$ . Glycine betaine (20  $\mu\text{mol}$ ) was added to one flask, kanamycin (100  $\mu\text{g cm}^{-3}$ ) to another, whilst the third flask was used as a control. The three flasks of *S. trochoidea* cultures were monitored over a 5 hr period. Every hour sub-samples were analysed, as previously described (Section 2.1.2) for DMS,

DMSPp, DMSPd, DMSOp and DMSOd concentrations. In addition, the algal cells were also examined under a light microscope throughout the sampling period to observe any visual effects due to the addition of either inhibitor. Kanamycin had no observable effect on cells or intracellular biogenic sulphur concentrations. In contrast, glycine betaine caused an immediate 5-fold increase in DMS concentrations which subsequently decline over time. This data is not presented in the results section, because it was not the focus of this study, though it is briefly mentioned in the discussion. On the basis of this early work, kanamycin was selected as an inhibitor and the grazing experiments using *T. longicornis* and *S. trochoidea* were repeated (in duplicate) with the inclusion of kanamycin ( $100 \mu\text{g cm}^{-3}$ ) in the media.

#### 4.2.4 Degradation of DMSP and DMSO in faecal pellets

A separate experiment to the main grazing work was performed to investigate the degradation rates of DMSP and DMSO in copepod faecal pellets. The copepod *T. longicornis* was starved for 48 hours then fed *S. trochoidea* under saturating conditions for 2 hours. The faecal pellets were collected into a glass Petri dish and replicate samples of 300 faecal pellets added to  $20 \text{ cm}^3$  vials containing autoclaved filtered seawater. The vials were crimp sealed and stored in the dark at  $14^\circ\text{C}$ . The vials were rotated periodically to maintain the pellets in suspension. Every 12 hours, 3 vials were randomly selected and analysed for DMSPp, DMSPd, DMSOp, DMSOd and DMS content.

Concentrations of the different sulphur species were determined sequentially from the same sample. The sample was filtered through two Millipore AP25 prefilters to collect the particulate fractions (DMSPp) and (DMSOp). The sample was purged for DMS

measurement and the sample then split for analysis of the dissolved fraction (DMSPd) and (DMSOd). All DMSP samples were added to glass crimp-top vials containing 2 cm<sup>3</sup> of 10 M NaOH and incubated for a minimum of 24 hours. DMSO was measured as previously described (Section 2.1.2).

### 4.3 Results

#### 4.3.1 Cultures of *S. trochoidea*

The concentrations of DMS, DMSPp, DMSPd, DMSOp, and DMSOd associated with *S. trochoidea*, prior to the addition of copepods, are shown in Table 4.2. The concentrations of DMS range from 3.3 to 4.8 nmol DMS dm<sup>-3</sup>. Concentrations of the particulate fractions of DMSPp and DMSOp display low variation between the different experiments, ranging from 2143 to 2521 nmol DMSPp dm<sup>-3</sup> and 240 to 374 nmol DMSOp dm<sup>-3</sup>. On a per cell basis, this represents an overall average concentration of 1.11 pmol DMSPp cell<sup>-1</sup> and 0.16 pmol DMSOp cell<sup>-1</sup>. These values are similar to DMSPp and DMSOp levels recorded in healthy exponentially growing cells (1.38 and 0.15 pmol cell<sup>-1</sup> respectively) in Chapter 3 (Section 3.3.1). In the extracellular pool, concentrations of DMSPd range from 24.5 to 60 nmol dm<sup>-3</sup> and levels of DMSOd range from 10.1 to 39.1 nmol dm<sup>-3</sup>. For both DMSPd and DMSOd, the highest concentrations are associated with the inclusion of antibiotics. Therefore the addition of antibiotics to cultures of *T. longicornis* produced an approximate two-fold increase in the levels of DMSPd and DMSOd.

**Table 4.2** Cellular concentrations of the methylated sulphur DMS, DMSPp, DMSPd, DMSOp, DMSOd in *S. trochoidea* cultures prior to the introduction of copepods.

Experiment		DMS (nmol dm <sup>-3</sup> )	DMSP (nmol dm <sup>-3</sup> )		DMSO (nmol dm <sup>-3</sup> )		Cell conc. per cm <sup>-3</sup>
			Particulate	Dissolved	Particulate	Dissolved	
<i>Acartia clausi</i>	a.	4.1	2594.6	39.5	292.7	27	2204
	b.	3.9	2143.3	32.6	266.7	17.4	2084
<i>T. longicornis</i>	a.	3.3	2621.4	32.9	374.6	16.8	2290
	b.	4.1	2280.5	24.5	249.1	10.1	2104
<i>T. longicornis</i> + antibiotics	a.	4.5	2187.4	60.0	240.6	39.1	2157
	b.	4.8	2535.9	55.0	285.9	35.8	2097

### 4.3.2 Grazing rates and DMSPp and DMSOp concentrations

The decrease in cell concentration for the experiment are shown in Table 4.3. The values were calculated by plotting algal cell density against incubation time and deriving the decrease in cell numbers per hour from the slope of a linear regression. The decrease in cell numbers indicated that both *A. clausi* and *T. longicornis* copepods grazed upon *S. trochoidea* throughout the 3 hour experimental period, and there was no evident adverse effect on grazing rates from the inclusion of antibiotics in the additional experiment. The average grazing rates for *A. clausi* were 111 cells copepod<sup>-1</sup> hr<sup>-1</sup>, compared to *T. longicornis* of 123 cells copepod<sup>-1</sup> hr<sup>-1</sup> and *T. longicornis* + antibiotics of 117 cells copepod<sup>-1</sup> hr<sup>-1</sup>.

**Table 4.3** Changes in DMSPp, DMSOp concentrations and cell density during the experimental period.

Experiment		Decrease in DMSPp (nmol dm <sup>-3</sup> hr <sup>-1</sup> )	Decrease in DMSOp (nmol dm <sup>-3</sup> hr <sup>-1</sup> )	Decrease in cell concentration (cells cm <sup>-3</sup> hr <sup>-1</sup> )
<i>A. clausi</i>	a.	100.5	39.9	116.3
	b.	93.6	21.7	105.1
<i>T. longicornis</i>	a.	106.9	47.6	121.2
	b.	102.1	35.04	124.9
<i>T. longicornis</i> + antibiotics	a.	99.0	38.4	112.9
	b.	105.5	33.2	120.4

The grazing rates (decrease in number of cells per hour) can also be calculated as  $\mu\text{g C}$  copepod<sup>-1</sup>. This is indirectly calculated by using the carbon:cell volume relationship reported by Menden-Deuer and Lessard (2000) for dinoflagellates:

$$\text{cell carbon (pg)} = 0.760 \times \text{cell volume } (\mu\text{m}^3)^{0.819}$$

The cell volume of *S. trochoidea* is  $8167 \mu\text{m}^3$  assuming volume =  $\pi d^2 l / 6$  ( $d$ =diameter,  $l$ =length) (see Section 3.2). The cell carbon content of *S. trochoidea* therefore equates to  $1215 \text{ pg C cell}^{-1}$ . To calculate the amount of carbon ingested per copepod during the grazing experiment, the total decrease in algal cells is divided by the number of copepods and multiplied by 0.001215 to provide  $\mu\text{g C ingested copepod}^{-1}$  (Table 4.4).

The average quantity of carbon assimilated at the end of the grazing period for *A. clausi* was  $2.02 \mu\text{g C copepod}^{-1}$  and *T. longicornis* was  $2.25 \mu\text{g C copepod}^{-1}$ . Table 4.4 compares these values to ingestion rates obtained for *T. longicornis* and *Acartia* sp. obtained in the marine environment for a 24 hour period (Kiørboe et al., 1985). Whilst copepod feeding rates obtained in the laboratory are not directly comparable to *in situ* feeding rates, they provide an indication on the quantity of carbon consumed relative to its daily requirement.

**Table 4.4** Carbon ingestion rates for *A. clausi* and *T. longicornis* during grazing on *S. trochoidea* and compared with values from Kiørboe (1985). It should be noted that the *Acartia* individuals in Kiørboe (1985) were *A. longiremis* and *A. tonsa*.

Experiment	Decrease in cell conc. (per $\text{cm}^{-3} \text{ hr}^{-1}$ )	No. of cells assimilated per copepod	Carbon per copepod $\mu\text{g C copepod}^{-1}$	$\mu\text{g C ingested copepod}^{-1} \text{ day}^{-1}$ (Kiørboe 1985)
<i>A. clausi</i> a.	116.3	1,745	2.12	0.36 – 0.54
b.	105.1	1,577	1.92	
<i>T. longicornis</i> a.	121.2	1,818	2.21	0.27 – 3.1
b.	124.9	1,874	2.28	
<i>T. longicornis</i> a.	112.9	1,694	2.06	0.27 – 3.1
+ antibiotics b.	120.4	1,806	2.20	

The decrease in *S. trochoidea* cell numbers due to grazing pressure was concomitant with a decline in DMSPp and DMSOp concentrations (Table 4.3). Concentrations of DMSPp

declined by an average of 96 and 104 nmol DMSPp dm<sup>-3</sup> hr<sup>-1</sup> in the presence of *A. clausi* and *T. longicornis* respectively. Concentrations of DMSOp declined by an average of 30 and 41 nmol DMSPp dm<sup>-3</sup> hr<sup>-1</sup> in the presence of *A. clausi* and *T. longicornis* respectively. Whilst DMSPp decreased at rates 2-3 times higher than for DMSOp, initial concentrations of DMSPp are much higher with the consequence that there is relatively little fluctuation in the DMSP: DMSO ratio per cell (average  $10.6 \pm 0.5$ ).

#### 4.3.3 Assimilation of DMSP into copepod body tissues

Prior to the grazing experiments, neither *A. clausi* nor *T. longicornis* had detectable quantities of DMSPp within their body tissues. Following the grazing on the DMSP-producing *S. trochoidea* and after they had voided their guts for two hours, DMSPp was detected in the body tissues of both *A. clausi* and *T. longicornis*. Average concentrations of DMSP in *A. clausi* were 0.19 nmol DMSP copepod<sup>-1</sup>, compared to *T. longicornis* which were 0.54 nmol DMSP copepod<sup>-1</sup> (Table 4.5). These values are compared to Tang et al. (1999), although the copepods in Tang's study were collected from the field.

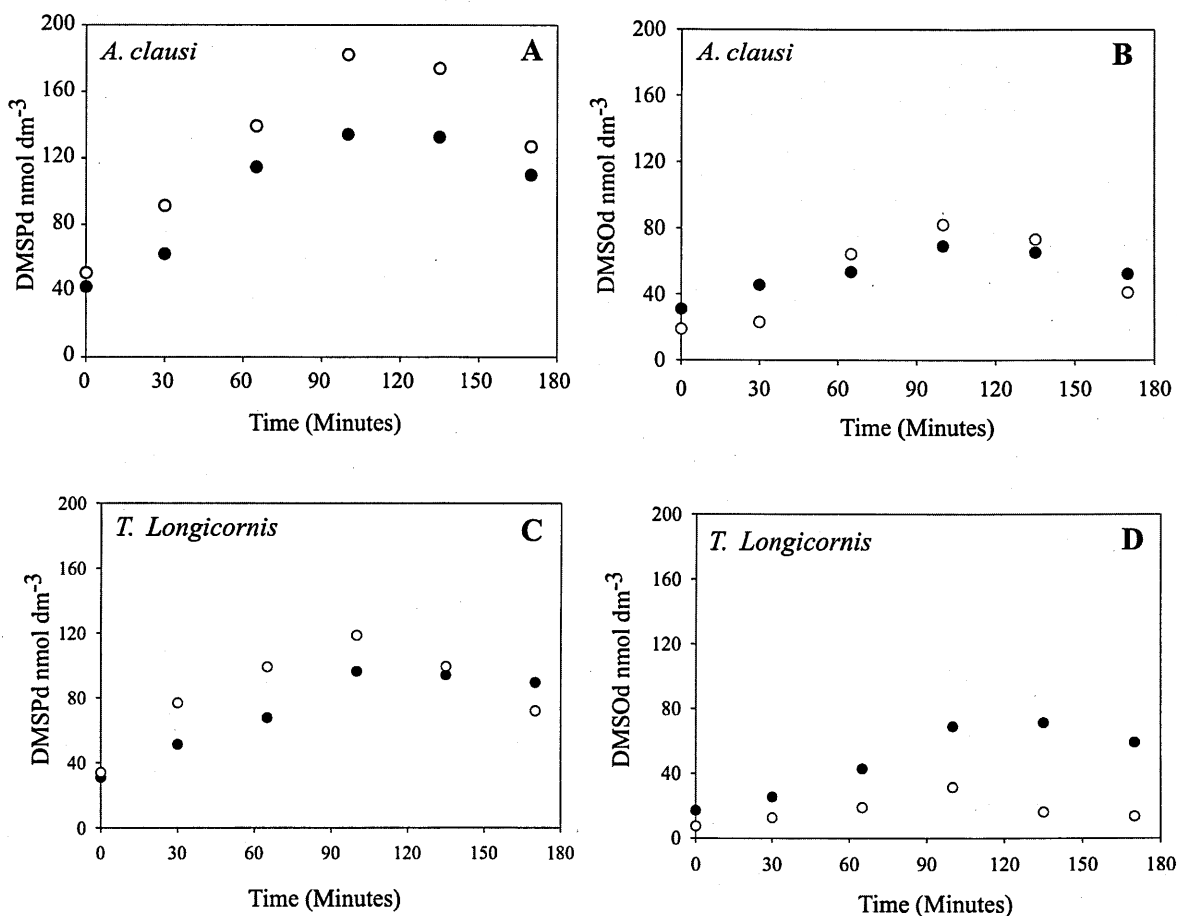
**Table 4.5** Concentration of DMSP assimilated by *A. clausi* and *T. longicornis* after grazing on *S. trochoidea*, compared with results from Tang (1999).

Experiment	DMSP assimilated nmol DMSP copepod <sup>-1</sup>	Tang (1999) nmol DMSP copepod <sup>-1</sup>
<i>Acartia clausi</i> a.	0.26	0.02
b.	0.13	
<i>T. longicornis</i> a.	0.46	1.03
b.	0.62	



#### 4.3.4 Release of DMSP and DMSO into solution

The effect of grazing on DMSPd concentrations was similar when either *A. clausi* or *T. longicornis* were used as grazers. An initial increase in DMSPd levels was observed up until approximately 90 minutes after the onset of grazing (Figure 4.5).

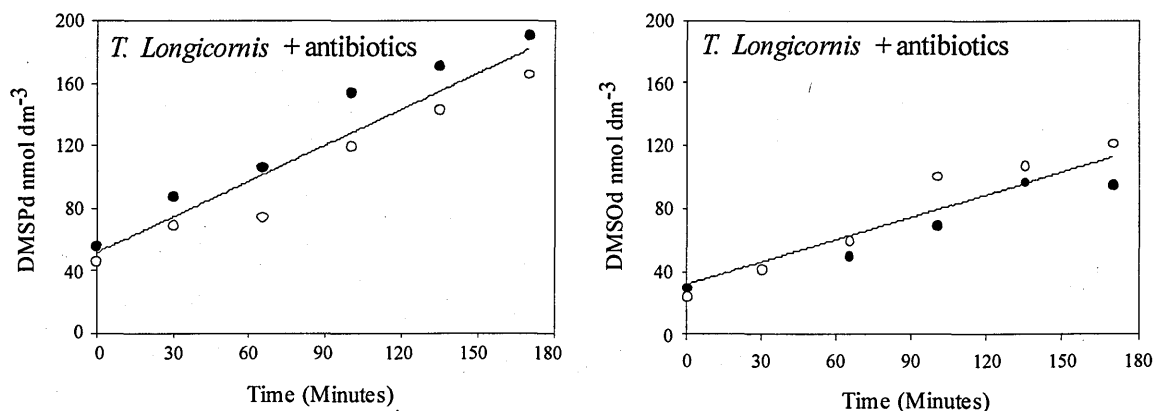


**Figure 4.5** Concentrations of DMSPd (Fig 4.5A and C) and DMSOd (Fig 4.5B and D) during grazing on the dinoflagellate *S. trochoidea* by the copepods *A. clausi* (A and B) and *T. longicornis* (C and D). The black and white symbols refer to duplicate experiments.

The initial rate of DMSPd increase was higher in *A. clausi* cultures (70 nmol dm<sup>-3</sup> hr<sup>-1</sup>) compared to *T. longicornis* (45 nmol dm<sup>-3</sup> hr<sup>-1</sup>). A maximum concentration of DMSPd observed for *A. clausi* and *T. longicornis* was 182 and 118 nmol dm<sup>-3</sup> respectively.

Subsequently, levels of DMSPd declined although concentrations never reached their pre-grazing concentrations. The effect of grazing on DMSOd concentrations followed a similar trend as observed for DMSPd levels in the presence of both *A. clausi* and *T. longicornis*. An initial increase in DMSOd levels was observed up until approximately 90 minutes of grazing pressure (Fig. 4.5). This initial rate of DMSOd increase was also higher in *A. clausi* cultures ( $54 \text{ nmol DMSOd dm}^{-3} \text{ hr}^{-1}$ ) compared to *T. longicornis* ( $32 \text{ nmol DMSOd dm}^{-3} \text{ hr}^{-1}$ ). A maximum concentration of DMSOd observed for *A. clausi* and *T. longicornis* was 82 and 71  $\text{nmol DMSOd dm}^{-3}$ .

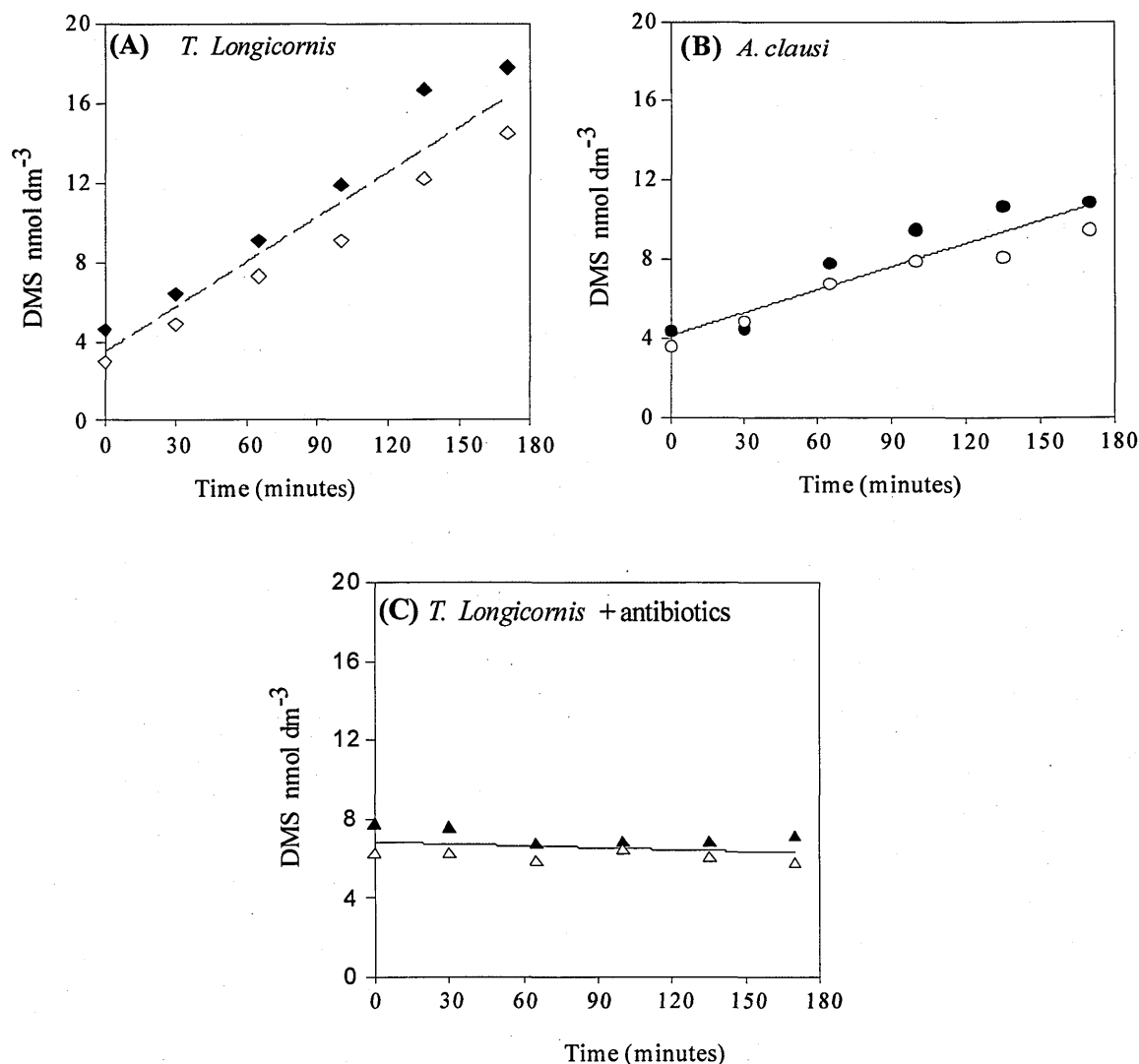
On all occasions of *A. clausi* and *T. longicornis* grazing on *S. trochoidea*, DMSPd and DMSOd concentrations declined after ~2 hours of grazing and this was hypothesised to be a result of bacterial metabolism. To examine this more closely, antibiotics were added to the media when *T. longicornis* grazed on *S. trochoidea*. Results clearly show that both DMSPd and DMSOd continued to increase in the presence of antibiotics (Fig. 4.6). The rate of increase for DMSP was  $46.1 \text{ DMSPd dm}^{-3} \text{ hr}^{-1}$  ( $y=0.768x+51.22$ ), whilst DMSOd concentrations increased at  $28.6 \text{ nmol DMSOd dm}^{-3} \text{ hr}^{-1}$ , ( $y=0.48x+31.8$ ).



**Figure 4.6** Concentrations of DMSPd and DMSOd in a repeat of the experiment shown in Fig. 4.5C & D, with the addition of the antibiotic kanamycin in the media. The black and white symbols refer to duplicate experiments and the trendline is an average of the two experiments and is used in Section 4.4.6 to calculate a budget for DMSP during grazing.

### 4.3.5 DMS production

A steady increase in DMS concentrations was observed during all grazing experiments when no antibiotics were added in the media. When *A. clausi* were used as grazers, DMS concentrations increased at an average rate of  $2.4 \text{ nmol DMS dm}^{-3} \text{ hr}^{-1}$  ( $y=0.0387x + 4.16$ ), whilst the average DMS production rates associated with *T. longicornis* were  $4.5 \text{ nmol DMS dm}^{-3} \text{ hr}^{-1}$  ( $y=0.0754x + 3.5$ ) (Fig. 4.7). The addition of antibiotics during a repeat of the grazing experiments with *T. longicornis* had a significant effect on DMS levels as no increase in DMS concentrations was observed throughout the experiment on both occasions when antibiotics were added (Fig. 4.7).



**Figure 4.7** Production of DMS during the grazing on the dinoflagellate *S. trochoidea* by (A) *T. longicornis*; (B) *A. clausi*; (C) *T. longicornis* + antibiotics. Results are shown for duplicate experiments with a trendline for the average concentration.

#### 4.3.6 Excretion via faecal pellets

At the end of the grazing experiment, after the copepods had been removed, faecal pellets were collected from the bottom of the experimental vessel. The total quantity of faecal pellets recovered provided an estimate of faecal pellets produced per copepod (Table 4.6). DMSP concentrations were quantified in triplicates of 50 faecal pellets and calculated to

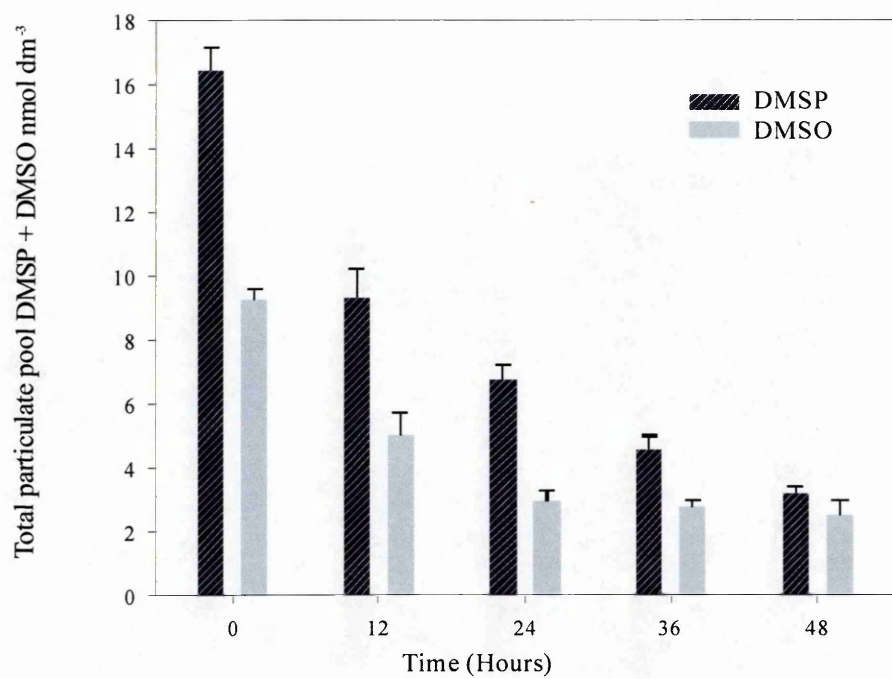
DMSP per faecal pellet. The DMSP concentration in *T. longicornis* faecal pellets (0.49 pmol DMSP faecal pellet<sup>-1</sup>) was ~4 times greater than for faecal pellets of *A. clausi* (0.12 pmol DMSP faecal pellet<sup>-1</sup>).

**Table 4.6** Concentration of DMSP in copepod faecal pellets and as a percentage of ingested DMSP that was subsequently excreted.

Experiment	DMSP excreted pmol DMSP pellet <sup>-1</sup>	No. of pellets copepod <sup>-1</sup>	% of assimilated DMSP that was excreted
<i>Acartia clausi</i> a.	0.14	10	0.07
b.	0.09		0.05
<i>T. longicornis</i> a.	0.46	9	0.2
b.	0.52		0.22

The percentage of ingested DMSP exported as faecal material was calculated from the quantity of DMSP ingested and defecated per copepod. The quantity of DMSP ingested per copepod is calculated from the number of cells assimilated per copepod (Table 4.4) and the DMSP content of *S. trochoidea* (1.11 pmol DMSP cell<sup>-1</sup>). The quantity of DMSP defecated is calculated from the quantity of faecal pellets produced per copepod and the DMSP content faecal pellet<sup>-1</sup> (Table 4.6).

In an independent experiment, *T. longicornis* faecal pellets were incubated over 48 hours to investigate the rate of decrease for DMSPp and DMSOp after grazing on *S. trochoidea*. Analysis was performed for DMSP (particulate and dissolved), DMSO (particulate and dissolved) and DMS. Levels of DMS, DMSOd and DMSPd were beneath detection limit and results are only shown for DMSPp and DMSOp (Fig. 4.8). The most rapid decrease of DMSPp and DMSOp occurred within the first 12 hours, with a 36 % and 40 % decrease, respectively. However, whilst DMSPp concentrations continue to decline over the 48 hours, DMSOp concentrations level off after 24 hours of incubation.



**Figure 4.8** Decrease in the concentrations of DMSPp and DMSOp within *T. longicornis* faecal pellets.

## 4.4 Discussion

### 4.4.1 Assimilation of DMSP into copepod body tissue

The assimilation of DMSPp in the body tissues of *A. clausi* and *T. longicornis* after grazing on the DMSP-producing dinoflagellate *S. trochoidea* suggests that accumulation by higher trophic organisms is highly dependent on diet. A similar laboratory experiment also found DMSPp concentrations within *T. longicornis* increased when fed the DMSP-producing *Tetraselmis impellucida* compared to the DMSP-poor *Dunaliella tertiolecta* (Tang et al., 1999). The dependence of DMSP accumulation on diet composition has also been revealed for larger organisms such as fish. Studies on DMSP in the muscle tissue of juvenile menhaden *Brevoortia tyrannus* reveal concentrations are typically  $< 200 \text{ nmol g}^{-1}$ , however after feeding on DMSP-producing algae they can reach up to  $820 \text{ nmol g}^{-1}$  (Hill and Dacey, 2006).

The concentrations of DMSP measured in *A. clausi* and *T. longicornis* during this study can also be compared to concentrations of DMSP within copepods collected from the marine environment (Table 4.5). Tang (1999) reported on the DMSPp content of five copepods that were captured from the marine coastal environment. Concentrations of DMSPp per copepod varied from  $0.01 \text{ nmol DMSPp copepod}^{-1}$  for *Paracalanus crassirostris*;  $0.02 \text{ nmol DMSPp copepod}^{-1}$  for *Acartia tonsa*; and  $1.03 \text{ nmol DMSPp copepod}^{-1}$  for *Temora longicornis*. During this study, grazing on *S. trochoidea* (i.e. a DMSP-rich diet) increased DMSPp concentrations in the body tissues of *A. clausi* by an order of magnitude greater than concentrations observed in the environment by Tang (1999). However in comparison to the DMSPp values reported by Tang (1999) for *T. longicornis*, the DMSPp values reported in this study ( $0.46\text{--}0.62 \text{ nmol DMSPp copepod}^{-1}$ ) were lower. The reason behind

this is not clear and it could be due to a physiological factor *e.g.* the rate at which DMSP is processed, or simply reflect the lower end of the range of DMSP concentrations in *T.*

*longicornis* body tissues.

During this study, no prolonged measurements were made on the DMSP concentrations within copepod body tissues to see if it remains at an elevated level or dissipates back to normal. Studies on menhaden fish revealed that after accumulating about one-third of their ingested DMSP into their tissues, DMSPd is subsequently lost gradually into the environment over a period of  $\geq 2$  weeks (Hill and Dacey, 2006). A similar period of time was also reported for carp, and rainbow trout based on a concentration of DMSP within body tissues of  $30 \text{ nmol g}^{-1}$  (Iida and Tokunaga, 1986). Tang et al. (2000) reviews the possible mechanisms by which DMSP is lost from copepods and suggests that DMSPd is released from their body and tissues with no chemical transformation (*i.e.* demethylation of DMS production).

#### 4.4.2 Release of DMSP and DMSO into solution

Grazing stimulated an increase in DMSPd and DMSOd during all experiments. Higher rates of DMSPd and DMSOd were observed in the presence of *A. clausi* compared to *T. longicornis*. A potential reason for this is the influence of the predator:prey size relationship on the quantity of material released to solution. Therefore the smaller the predator:prey ratio, the increased amount of DOM released into solution as the predator has to handle a relatively larger prey organism (Møller, 2005). Hansen et al. (1994) evaluated the average size of the predatory copepod compared to its prey and determined that the ratio ranges from 10:1 to 30:1 (optimum average of 18:1), with size being



determined as equivalent spherical diameter (ESD). The ESD of *A. clausi*, *T. longicornis* and *S. trochoidea* are 533, 388 and 24  $\mu\text{m}$  respectively, which convert to a size ratio of 16.7 and 22.2 for *A. clausi* and *T. longicornis* respectively. This indicates that whilst the lower predator:prey size ratio associated with *A. clausi* might reflect the greater increase of DMSPd and DMSOd compared to *T. longicornis*, both copepods were feeding on prey that were near to the optimum ratio of 18:1, as proposed by Hansen et al. (1994).

It should also be noted that the release of algal material due to sloppy feeding is just one possible mechanism responsible for releasing algal-DMSPp and DMSOp into solution during grazing. The dissolution of faecal material also contributes to the dissolved pool (Tang et al., 2000a; Møller et al., 2003) and the relative contribution of the two pathways to the dissolved pool is not always apparent. However, it is likely that dissolution of organic matter from faecal pellets would have an intrinsic time delay of  $\sim 30$  min due to the gut passage time (Dam and Peterson, 1988). During the grazing by *Acartia clausi* and *Temora longicornis* on *S. trochoidea* the increase in DMSPd and DMSOd occurred immediately. It is therefore considered likely that sloppy feeding, and potentially other mechanical mechanisms e.g. swimming, are responsible for the release of algal material during this study.

After DMSP and DMSO were released into solution from algal cells they were rapidly metabolised by bacteria, as inferred indirectly through the addition of antibiotics (Fig. 4.6). There is widespread evidence for the metabolism of DMSP by bacteria in the literature (Kiene and Taylor, 1988; Visscher and van Gemerden, 1991; Dodd et al., 2006). In this experiment, the addition of kanamycin caused a difference in DMSPd concentrations of  $103 \text{ nmol dm}^{-3}$ . Previous rates of DMSPd consumption have been calculated at  $\sim 4 \text{ nmol}$

$\text{dm}^{-3} \text{hr}^{-1}$  in the Duplin River (Kiene and Service, 1991). During a coccolithophore bloom in the North Sea, DMSPd consumption rates were  $20 \text{ nmol dm}^{-3} \text{hr}^{-1}$  (surface waters) and  $40 \text{ nmol dm}^{-3} \text{hr}^{-1}$  (sub-surface waters) (Zubkov et al., 2002).

The increase of DMSOd during the grazing experiments supports the observation in Chapter 3 that algal cells are a major source of DMSOd in the marine environment. The results from this Chapter show that grazing can further increase the DMSOd pool in the marine environment. The quantity of DMSOd released after 3 hours, as estimated through the addition of antibiotics, was  $71.9 \text{ nmol dm}^{-3}$  (Fig 4.6). In comparison with environmental conditions, the microbiological production of DMSO ranged from  $0.07$  to  $0.33 \text{ nmol dm}^{-3} \text{day}^{-1}$  during incubations of natural seawater samples in the Sargasso Sea (del Valle et al., 2007). The results from the grazing experiments and Chapter 3 suggest that the release of DMSO from algal cells can be a major source of DMSOd in the marine environment.

A general observation on the release of algal-DMSP and DMSO, with subsequent microbial consumption is that the effect may only be obvious on short-time scales *e.g.* hours. Grazing experiments where measurements exceed 1-2 hours may miss this pulse of DMSPd increase and conclude that meso-zooplankton had no effect on DMSPd concentrations. This could have occurred in the mesocosm experiments investigating *Emiliania huxleyi* where measurements were taken daily (Levasseur et al., 1996) and also during incubations with copepods when measurements were taken every 3 hours (Christaki et al., 1996).

An additional noteworthy observation made during the preliminary work of this grazing experiment relates to the use of inhibitors. The addition of glycine betaine to *S. trochoidea* caused a 5-fold increase in DMS concentrations which subsequently dissipated over the following five hours. This contrasts with previous reports which have used at a similar range of concentrations (1 to 50  $\mu\text{mol}$ ) and reported no similar effects (Kiene and Gerard, 1995). The rapid increase suggests that instead of competitively inhibiting DMSPd uptake by bacteria, the additional nutrient source induced DMSP-associated microbial metabolism. The resulting DMS production could have either been due to a switch from DMSP demethylation to DMSP-lyase pathway, or due to conversion of DMSO to DMS.

#### 4.4.3 DMS production

An increase in DMS concentrations of 2  $\text{nmol dm}^{-3} \text{ hr}^{-1}$  and 4  $\text{nmol dm}^{-3} \text{ hr}^{-1}$  was observed when *A. clausi* and *T. longicornis* grazed on *S. trochoidea* respectively. These rates of DMS production are similar to Dacey and Wakeham (1986) who observed an increase in  $\sim 200 \text{ nmol DMS dm}^{-3} \text{ day}^{-1}$ . During shipboard incubation grazing experiments with krill, DMS concentrations increased by 200  $\text{nmol dm}^{-3} \text{ hr}^{-1}$  (Kasamatsu et al., 2004).

Interestingly, the authors showed that whilst DMS increased due to krill grazing, during feeding by salps there was no effect on DMS levels. This was attributed to the sloppy feeding nature associated with krill, compared to salps which ingest entire cells (Kasamatsu et al., 2004). Kiene et al. (2000) proposes that the production of DMS depends upon the bacterial demand for sulphur relative to ambient levels of DMSPd. The increase in DMS concentrations during the experimental period suggests that the bacterial demand for DMSPd was satiated. This indicates that grazing by mesozooplankton can

decouple the microbial consumption of DMSPd from its production (*i.e.* algal exudates) over short-time intervals.

The addition of antibiotics caused a complete absence in the production of DMS. This finding indicates that within the confines of this experiment, DMS production is microbially mediated and implies that *S. trochoidea* does not produce DMS by itself (Fig. 4.2). From a microbial perspective, this result is perhaps unsurprising as the role of bacteria in the production of DMS has been recognised for several decades (Kiene and Visscher, 1987). However, the apparent inability of a dinoflagellate species to produce DMS has repercussions for the anti-grazing hypothesis, as proposed by Wolfe (2000). It suggests that the DMSP anti-grazing hypothesis will have to involve either free-living bacteria or microbes associated with the algal surface. Whilst the involvement of bacteria is still plausible, this has so far not been included in the research (Wolfe and Steinke, 1996; Steinke et al., 2002). It can be concluded that the microbial-mediation of DMS production associated with algal cultures requires careful confirmation. Further work should consider investigating whether other cultures of *S. trochoidea* and other phytoplankton cultures produce a similar result.

#### 4.4.4 Release of DMSP and DMSO in faecal pellets

An average concentration of DMSPp within the faecal pellets of *A. clausi* and *T. longicornis* in this study was 0.11 and 0.49 pmol DMSP faecal pellet<sup>-1</sup>. These values are at the middle of the range of DMSP concentrations previously reported for copepod faecal pellets of 0.033 pmol faecal pellet<sup>-1</sup> for *A. tonsa* (Tang, 2001) and 5 pmol faecal pellet<sup>-1</sup> for *Eurytemora affinis* (Kwint et al., 1996). The differences between DMSP concentrations could be due to natural variation and also methodological issues such as the time delay

before analysis and the dissolution of faecal material during the handling of the faecal pellets.

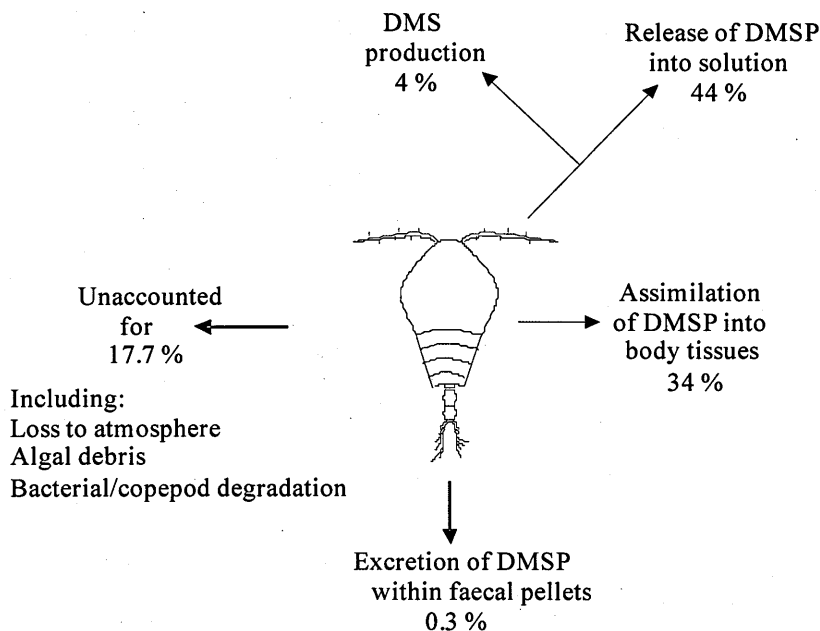
To look at the contextual relevance of DMSPp within faecal pellets, it is necessary to consider the faecal pellets on the same spatial scale as DMSPd, *i.e.* DMSP per  $\text{dm}^{-3}$  of faecal material. A comparison can be made assuming the global average for DMSPd of  $16 \text{ nmol dm}^{-3}$  (Kettle et al., 1999) and a faecal pellet DMSPp concentration of  $0.49 \text{ pmol DMSP pellet}^{-1}$  (Taken from *T. longicornis*, Table 4.6) with a faecal pellet volume of  $2.5 \times 10^5 \mu\text{m}^3$  (Chapter 7). This equates to a concentration gradient of 5 orders of magnitude from within a faecal pellet to the surrounding water column. The concentrated source of DMSP highlights how faecal pellets can act as ‘hotspots’ for microbial activity compared to the wider column, as proposed by Tang (2001). Scarratt et al. (2000) suggest that these microzones of high DMSP concentration account for the wide variation of half-saturation constants ( $K_m$  values) of bacteria for DMSP cleavage which range from nanomolar to micromolar levels (Ledyard and Dacey, 1994, 1996; Kiene et al., 1998). These values suggest that certain bacteria species are accustomed to high levels of DMSP that may be found surrounding algal cells or in detrital particles such as faecal pellets

One possible explanation for the lack of DMSPd or DMSOd detection is the microbial consumption of these compounds. The degradation of DMSPp or DMSOp could have occurred aerobically as reported for the dissolved fractions (Section 4.4.2). Alternatively, the degradation of DMSPp and DMSOp could have occurred anaerobically due to localised oxygen depletion within faecal pellets (Alldredge and Cohen, 1987). In support of potential anaerobic degradation of organic material, previous workers have isolated obligate anaerobic microbes *e.g.* methanogenic Archaea, from faecal pellets. Furthermore

the derivative compounds of DMSP (DMS, methanethiol, dimethylsulphide, 3-methylpropionate) are all potential metabolic substrates for methanogens. The anaerobic degradation of DMSP is examined in much greater detail in Chapters 6 and 7.

#### 4.4.6 Fate of algal-DMSP during grazing by *T. longicornis*

Using the estimates of the different sinks discussed so far for DMSPp, an overall budget can be created to represent the fate of algal-DMSPp during grazing (Fig. 4.9). The budget uses average concentrations from the duplicate experiments with *T. longicornis* grazing on *S. trochoidea*. A total loss of DMSPp from algal cells during the experiment ( $315 \text{ nmol dm}^{-3}$ ) is used to represent 100 %. For the sinks where a production rate is known *i.e.* DMS ( $4.5 \text{ nmol hr}^{-1}$ ) and DMSPd ( $46.1 \text{ nmol hr}^{-1}$ ), the total quantity produced at the end of the experimental period is used. The assimilatory and excretory pathways, which were previously reported as DMSPp copepod<sup>-1</sup> and DMSPp faecal pellet<sup>-1</sup> respectively, are converted to reflect the number of copepods used and the number of faecal pellets produced in the study.



**Figure 4.9** DMSP flux during grazing by *T. longicornis* on *S. trochoidea* at the end of three hour grazing experiment, showing percentage of DMSP removed from algae that ends up in the different pools.

The budget does not include DMSO values as no measurements were made on the copepod and faecal pellet sinks during the grazing experiments. Furthermore, it is not possible to directly construct a similar budget for *A. clausi* as no net value of DMSPp released into solution was calculated. However assuming that a similar pool of DMSPp is unaccounted for in *A. clausi*, a proportional value for DMSPd can be estimated (Table 4.7).

**Table 4.7** Different sinks for DMSPp released during grazing by *A. clausi* on *S. trochoidea*. To estimate the production of DMSPd, the unaccounted pool was assumed to be the same as *T. longicornis*.

Pool	Production	% Contribution
DMS	2.4 nmol hr <sup>-1</sup>	2.4
DMSPd	-	64.1
Assimilation	39 nmol copepod <sup>-1</sup>	13.4
Excretion	0.23	0.07
Unaccounted for	-	18

The data presented in Figure 4.9 and Table 4.7 contribute to the growing understanding of the diverse trophic role played by the grazing of copepods. The primary aim of grazing by heterotrophic organisms is to obtain nutrients for metabolism and energy. However the assimilation of organic material into grazers' body tissues is not a foregone conclusion as this study shows that grazing greatly enhanced the production of DMSPd. Prior to the introduction of copepods, 1.2 % of total DMSP (DMSPp+DMSPd) was found in the dissolved form. In comparison, an overall average of 7 % of total DMSP was in the dissolved phase for the algal cultures analysed in Chapter 3. Grazing increased the DMSPd pool to an average of 44 % and 64 % in *T. longicornis* and *A. clausi* respectively. Other studies have also reported similar increases in DMSPd. During ciliate grazing on *Isochrysis galbana*, 66 % of DMSPp released was recovered as DMSPd (Christaki et al., 1996). Similarly during grazing by *Calanus* sp. on *Thalassiosira gravida*, ~50 % of algal carbon was released into solution (Møller et al., 2003).

Whilst faecal material is a relatively minor sinks for algal-DMSP compared to the release from algal cells during sloppy feeding, DMSPp in faecal pellets are a significant components of the biogenic sulphur cycle. Field-based measurements have analysed



sinking particulate material for DMSPp content based on material collected in sediment traps deployed in the water column (Chapter 5, Table 5.1). To date, there have been no reports on DMSP concentrations within faecal pellets collected from the marine environment. In field-based measurements, the overall contribution of downwards flux of DMSPp to the standing stock was estimated at  $\leq 1\%$  which exceeds the 0.3 % of DMSP in excreted material for this study (discussed further in Section 5.1). The difference in values could reflect a greater concentration of DMSP within faecal pellets during intensive grazing studies or reflect the DMSPp in non-faecal sinking material in the water column.

An important point to consider is the relevance of the grazing experiment to environmental conditions. The experimental design is likely to have influenced results.

For example, within the experimental vessels the turbulence regimes are different to the field environment affect the copepods prey-detection ability (Roman and Rublee, 1980; Visser, 2001). Furthermore, the starvation of copepods prior to the experiment was ensure rapid grazing occurred, yet starved copepods have been shown to be more efficient feeders (Runge, 1980). In comparison with estimates on the quantity of carbon consumed per day by each species of copepod (Kiørboe et al., 1985), both copepods appeared to have satiated their daily nutritional requirement within the experimental period (Table 4.4).

## 4.5 Conclusion

During copepod grazing, concentrations of DMSP, DMSO and DMS reflected a combination of grazer, algae and bacteria activity. The addition of inhibitors indicated that the production of DMS associated with *S. trochoidea* was microbially-mediated. Furthermore, DMS production was considered to result from a decoupling between bacterial consumption and the release of DMSPp from algal cells. The grazing by copepods triggered this decoupling, however it is hypothesized that this is apparent on a short (<6 hour) timescale only. DMSOp was also released from algal cells due to grazing and it contributes to an ambient dissolved pool of DMSOd. Future work should investigate the subsequent fate of DMSOd and whether it represents a source for DMS over short-time scales. This study also revealed that copepod faecal pellets represent hotspots of DMSPp in the upper water column. Concentrations reached micromolar concentrations when calculated on a  $\text{dm}^{-3}$  of substrate scale. Therefore both algal cells (Chapter 3) and copepod faecal pellets (this study) represent micro-environments in the upper water column which have high concentrations of DMSP and DMSO in comparison to ambient levels. This is an important consideration for the activity of anaerobic microbes which is discussed in following Chapters.

## Chapter 5. DMS, DMSP and DMSO in Loch Creran

### 5.1 Introduction

The continental margins play a major role in marine biogeochemical cycling despite occupying only 7 % of the ocean surface and less than 0.5 % of the ocean volume. Due to the rapid turnover and the higher supply of nutrients from upwelling and river inputs, significantly higher rates of primary productivity occur in the coastal environments than in open oceans (Chen et al., 2003). The coastal margins also contribute to climatic forcing through the release of biogenic gases such as nitrous oxide (Bange et al., 1996), methane (Upstill-Goddard et al., 2000) and dimethylsulphide (DMS) (Liss et al., 1997). The continental shelf margins and upwelling areas are typically associated with higher concentrations of DMS than the open ocean, though there is also pronounced patchiness on a relatively small scale (Kettle et al., 1999). This mesoscale variability was referred to as 'DMS hotspots' during field research of DMS distribution on the European western continental margin and related to areas where DMS concentrations could be 40 % higher than the background average (Uher et al., 2000). These DMS hotspots were correlated with hydrographic features such as frontal systems along the Celtic Sea margin ( $4\text{--}10\text{ nmol dm}^{-3}$ ) and on the shelf as well as wind-driven upwelling along the Iberian margin ( $10\text{--}19\text{ nmol dm}^{-3}$ ).

In addition to the spatial variability, seasonal field studies and data compilations reveal a temporal component to DMS biogeochemistry particularly in coastal and shelf seas, with an accumulation of DMS during the summer months (Dacey et al., 1998; Kettle et al., 1999; Simo and Pedros-Alio, 1999; Uher et al., 2000). An overall trend of higher DMS

concentrations and sea-to-air fluxes with increasing primary production is typically observed (Andreae and Barnard, 1984). During a temporal study of DMS in the near-shore waters around Britain, the mean winter concentrations were  $0.1 \text{ nmol DMS dm}^{-3}$ , compared to an average summer concentration of  $6.9 \text{ nmol DMS dm}^{-3}$  (Turner et al., 1988). At mid-low latitudes, DMS concentrations can reach a maxima two months after its precursor dimethylsulphoniopropionate (DMSP), a phenomena known as the 'DMS summer paradox' (Simo and Pedros-Alio, 1999). The authors reach the same conclusion as Uher et al. (2000) in that to understand DMS biogeochemistry an integration of the couplings and decouplings of the biological, physical and chemical processes is required.

The complex interplay of physical, chemical and biological processes that drive DMS concentrations occur across a variety of trophic levels (Chapter 1, Fig. 1.5). DMS is derived from the bacteria- and phytoplankton-mediated enzymatic cleavage of the algal compound dimethylsulphoniopropionate (DMSP). Intracellular concentrations of DMSP vary between different phytoplankton species by four orders of magnitude (Chapter 3; Table 3.3). Despite the phytoplankton origin of DMSP, and the conversion to DMS, surface concentrations of DMS are generally poorly correlated to either chlorophyll *a* concentrations at a regional to global scale (Kettle et al., 1999; Simo and Vila-Costa, 2006) or in seasonal studies (Leck and Persson, 1996; Turner et al., 1996; Dacey et al., 1998). This is due to a number of reasons including the taxonomic-dependent production of DMSP, its intracellular physiological role, and the influence of biological processes on the conversion of DMSP to DMS *e.g.* grazing by higher trophic organisms and bacterial consumption (as discussed in Chapter 4). Furthermore, DMS itself is subject to a number of loss processes including microbial transformation, photo-oxidation and sea-air exchange. The microbial consumption of DMS is regulated by the bacterial nutrient

demand and microbial community composition, which can be inhibited by UV radiation (Slezak and Herndl, 2003; Toole et al., 2006). DMS photo-oxidation is dependent on incident solar radiation and sea-surface temperature (Toole et al., 2003; Toole et al., 2006).

The seasonal studies and field-based research investigating spatial and temporal changes in DMS and DMSP concentrations have frequently neglected to measure accompanying concentrations of dimethylsulphoxide (DMSO), although it is recognised as an important component of the sulphur biogeochemical cycle. Similar to DMSPp, the production of DMSOp in phytoplankton is taxon-related where it can represent ~ 20 % of intracellular methylated sulphur (DMSPp+DMSOp) (Chapter 3). In comparison to DMSPp, there have been far fewer reports on the seasonal variation of DMSOp in surface seawater and the possible factors that determine its ambient concentration. During an annual study of biogenic sulphur at Blanes Bay, Mediterranean, DMSOp concentrations reached a maximum of 11 nmol dm<sup>-3</sup> during early summer, with an annual range from 0.9 to 14 nmol dm<sup>-3</sup> (Simo and Vila-Costa, 2006). In Scottish coastal waters, DMSOp concentrations reached 20 nmol dm<sup>-3</sup> during spring and summer. Increased levels of DMSOp were concomitant with increases in DMSOd, DMS and chlorophyll *a* (Hatton et al., 2004).

Within the biogenic sulphur cycle, it was always considered that a proportion of DMSP produced in the surface waters was lost through sedimentation (Dacey and Wakeham, 1986; Simó, 2001). There are several pathways involved in the downwards flux of material: i) sinking of aggregated plant and animal remains; ii) detrainment of particles during pycnocline shallowing and iii) active downward transport caused by feeding, defecation, respiration and reproduction of migrating zooplankton and higher trophic organisms (Turley and Mackie, 1994). The laboratory experiments in Chapter 4 revealed

that on average 0.07 % and 0.3 % of DMSPp ingested by the copepods *A. clausi* and *T. longicronis* was excreted within faecal pellets. This supported the findings of previous laboratory grazing experiments, when Tang et al. (2001) demonstrated that 0.1 % of DMSP ingested was excreted during grazing by *A. tonsa*. A similar experiment found a much higher percentage (10-50 %) of the DMSP consumed by the copepod *Eurytemora affinis* during grazing on dinoflagellates was excreted as faecal pellets (Kwint et al., 1996).

Within the marine environment, the sedimentation flux of DMSP has been quantified on separate occasions in different marine locations (Table 5.1) and calculated as a percentage of the overall standing stock in the water column. Two of these study sites represent open ocean environments: the North Eastern Pacific, and the East Atlantic; the other two are shelf environments: the northern North Sea, and the Antarctic coastal environment. In the shelf environments, sedimentation rates of DMSPp are an order of magnitude higher and represent 1 % of the standing stock (Table 5.1). This reflects the elevated surface water biological activity and primary production associated with near-shore waters (Chen et al., 2003). The downward flux of DMSP can be compared with the total carbon downwards flux for two of the areas. In the North Sea coccolithophore bloom, the downwards flux of carbon, was calculated using  $^{234}\text{Th}$  disequilibria, at 9.5 to 48 mmol C m<sup>-2</sup> d<sup>-1</sup>, which equated to export rates of 7 to 96 % of carbon production (Foster and Shimmield, 2002). In the NE Pacific, the carbon flux was considerably less, represented 0.95 mmol C m<sup>-2</sup> d<sup>-1</sup> which equated to 0.8 % of the standing stock (Bates et al., 1994).

**Table 5.1** Downwards flux measurements of DMSPp reported as an overall concentration and also as the % contribution of DMSPp in sedimenting material relative to the overall integrated standing stock of DMSP in the water column.

Location	Downwards flux of DMSP $\mu\text{mol m}^{-2} \text{d}^{-1}$ (depth)	Accumulation rate % $\text{d}^{-1}$	Reference
NE Atlantic Ocean	5.7 (200 m)	0.1%	Corn (1994)
North Sea	8.4 – 16 (30-40m)	1%	Hatton (2002)
Coastal Antarctic	Not provided	1%	Daly & DiTullio (1996)
NE Pacific	0.18 – 2.65 (100-200m)	0.1%	Bates (1994)

The low rates of DMSP sedimentation support the general concept that DMSP and DMS represent an extremely labile source of carbon for bacteria with a high mass flux throughout the microbial food web (Kiene and Linn, 2000). Within sinking particulate material, DMSP degradation may occur by aerobic (Taylor and Gilchrist, 1991; Gonzalez et al., 1999) or anaerobic (Kiene and Taylor, 1988) bacterial metabolism, as the high bacterial respiration rates that occur on sinking particulate matter may facilitate oxygen depletion within micro-niches (Alldredge and Cohen, 1987; Turner, 2002; Grossart et al., 2003). However, in contrast to the loss of DMSP and DMS, concentrations of DMSO increased during the incubation of material recovered from sediment traps in the middle of a coccolithophore bloom in the North Sea. This was considered to result from the microbial degradation of DMSP to DMS and subsequent oxidation to DMSO (Hatton, 2002). It is not apparent whether the production of DMSO was occurring via aerobic or anaerobic pathways. This transformation of DMSP and DMS to DMSO may explain why DMSO concentrations have been reported at levels exceeding  $1.5 \text{ nmol dm}^{-3}$  at depths of over 1000 m in the equatorial Pacific and Arabian Sea (Hatton et al., 1998; Hatton et al., 1999), whereas DMS and DMSP are usually confined to the euphotic zone.

This study investigated the dynamics of DMS, DMSP and DMSO on the west coast of Scotland in Loch Creran, looking at the surface concentrations, the downwards flux, and changes during the sedimentation. Loch Creran has been the study site and the focus of many scientific studies dating from the 1970's to the present day. Some of the earliest work looked at sedimentation in sea-lochs including Loch Creran (Ansell, 1974), whilst more recent published work has modelled the nutrient dynamics in Loch Creran to look at the effects of the implanted fish farm (Laurent et al., 2006). The published work includes work on carbon and nutrient budgets (Ansell, 1974; Tett et al., 1975; Tyler, 1983), the physical exchanges and dynamics of phytoplankton (Tett, 1986) and the ecology of phytoplankton (Tett and Wallis, 1978; Tett et al., 1981; Gowen et al., 1983; Tett, 1986). In addition, PhD theses by Jones (1979); Tyler (1983); Fehling (2004) and Pete (2007) also provide much information on the hydrography, nutrient profiles and phytoplankton composition of the loch.

The above work has shown that the water in Loch Creran has a typical residence time of a week due to flushing of water from Loch Linhe driven by a fjordic-estuarine circulation (Tett, 1986). The brackish layer in Loch Creran extends down to 8 m. Below this depth, the salinity fluctuations rarely go below 30, whilst on the surface concentrations typically exceed 25 (Tett and Wallis, 1978). The lower salinity is a result of freshwater run-off and river discharge, however neither of these fresh water inputs are thought to have a detrimental effect on nutrient concentrations (Tett and Edwards, 2002). At 8 m depth in the water column the lower limit of the euphotic zone is also found (Jones, 1979). The combination of the clear water in the upper 8 m, persistent haline stratification and surface water concentrations provides sufficient illumination for phytoplankton growth throughout the majority of the year (Tett and Wallis, 1978). In addition, due to its shallow nature,



Loch Creran does not house populations of large copepods such as *Calanus finmarchicus* or euphausiids such as *Thysanoessa raschii* which could control phytoplankton populations by grazing (Tett et al., 1985). With regards to previous published work in biogenic sulphur concentrations in Loch Creran report, the concentrations of DMS and DMSO throughout the period of one year have been reported (Hatton et al., 2004).

The objectives of this study were:

- i) analyse DMS, DMSP and DMSO throughout the Spring bloom;
- ii) quantify the downwards flux of these compounds;
- iii) investigate the conversions between DMS, DMSP and DMSO in the sinking material.

## 5.2 Methods

### 5.2.1 Site selection

The sampling site was located in Loch Creran, approximately 1 hour travel time from Dunstaffnage Marine Laboratory on the small research vessel *RV Seol Mara*. For a description of Loch Creran and the surrounding environment, see Chapter 2 (Section 2.4). To investigate the context of the sampling site in relation to other locations in Loch Creran and Loch Linnhe, a transect was conducted on 24<sup>th</sup> May 2005, consisting of 8 stations (Fig. 5.1). At each station, surface seawater was collected using a NIO bottle for the analysis of DMS, DMSPp, DMSPd and DMSOd and ancillary data of depth, salinity and temperature were recorded.

A fortnightly sampling programme of seawater analysis and sediment trap deployment ran from March to June 2005 within Loch Creran based at Station 3 (Fig. 5.1). The Station is situated in the deepest part of the loch, situated near the seaward end of South Shian. The site was selected because it represented a deep water location in a nearby location that allowed deployment and retrieval of the sediment trap using the *RV Seol Mara* at all times of the year.

### 5.2.2 Water column analysis

Water samples were collected from two depths in the water column: surface water (top 1 m) and 20 m depth, using a 1 litre NIO bottle attached to a winch. Immediately after recovery, water from NIO bottles was decanted into pre-rinsed 500 cm<sup>3</sup> glass-stoppered bottles using a short piece of silicon tubing. Bottles were filled to overflowing and sealed

with glass-stoppered bottles, ensuring no headspace and minimal gas loss throughout the procedure. The bottles were then stored on-deck, in the dark at ambient seawater temperature, for transport back to the laboratory. The analysis of water column samples occurred within 2-3 hours of sample collection. Analysis for DMS, DMSPp, DMSPd, DMSOp, DMSOd, POC and chlorophyll *a* was performed following the same routine as described in Section 2.1.2. Samples for surface seawater and 20 m seawater were immediately analysed for DMS and samples prepared for the future analysis of DMSPp, DMSPd, DMSOp and DMSOd. This was followed by the analysis of trap material for DMS concentrations and preparation of DMSPp, DMSPd, DMSOp, DMSOd, and POC for future analysis. Finally the seawater samples were filtered for POC and chlorophyll *a* analysis.

#### Particulate organic carbon (POC) analysis

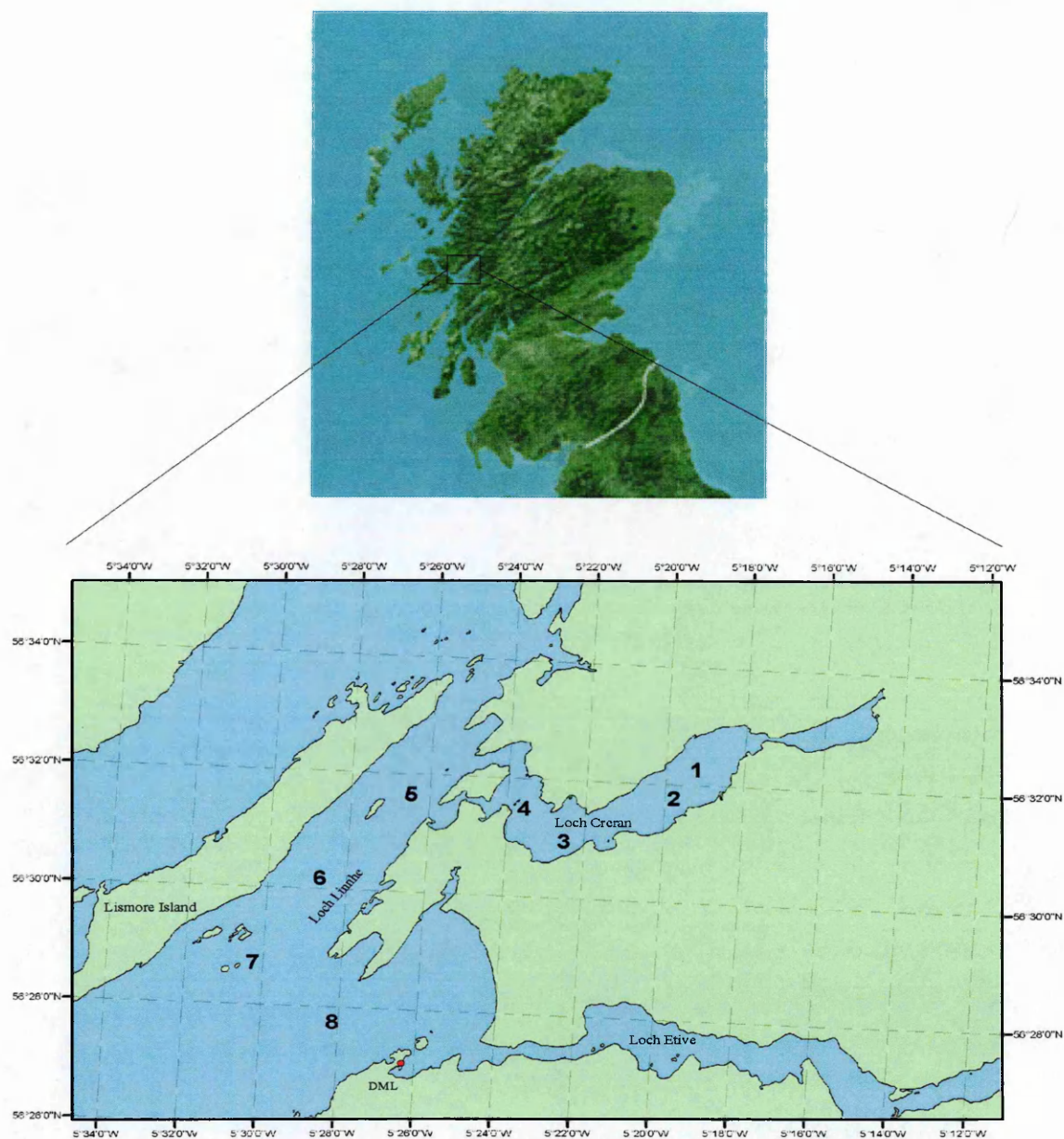
100 cm<sup>3</sup> of seawater sample was filtered onto a glass fibre filter (Whatman GF/F, 13 mm diameter, pre-ashed for 24 hours at 450 °C, 0.7 µm nominal porosity) and stored frozen at -20 °C. Prior to analysis the filters were oven dried at 60 °C overnight and folded into tin capsules. Analysis for particulate carbon was performed on a ANCA NT prep system coupled with a 20-20 Stable Isotope Analyser (PDZ Europa Scientific Instruments). The calibration was performed using an Isoleucine solution (L-Isoleucine δ-calibrated, Europa STD, 1 µgN/µl and 6 µgC/µl) and the standards were dried out in tin caps prior to usage.

#### Chlorophyll *a* analysis

500 cm<sup>3</sup> of seawater sample was filtered on a 25 mm glass fibre filter (Type A/E, Pall Corporation) and stored frozen at -20 °C in eppendorf tubes. Prior to analysis, the filters were thawed and the pigments extracted using 4 cm<sup>3</sup> of 90 % acetone overnight at 4 °C.

The filters were then sonicated for 1 min, centrifuged (3000 rpm for 5 min), and the supernatant analysed for chlorophyll using a simple isocratic HPLC method with reverse phase separation, a C-18 column and 90 % Methanol, 10 % Acetone eluant (Mantoura et al., 1997). The chlorophyll *a* results have been kindly provided by Pete (2007).

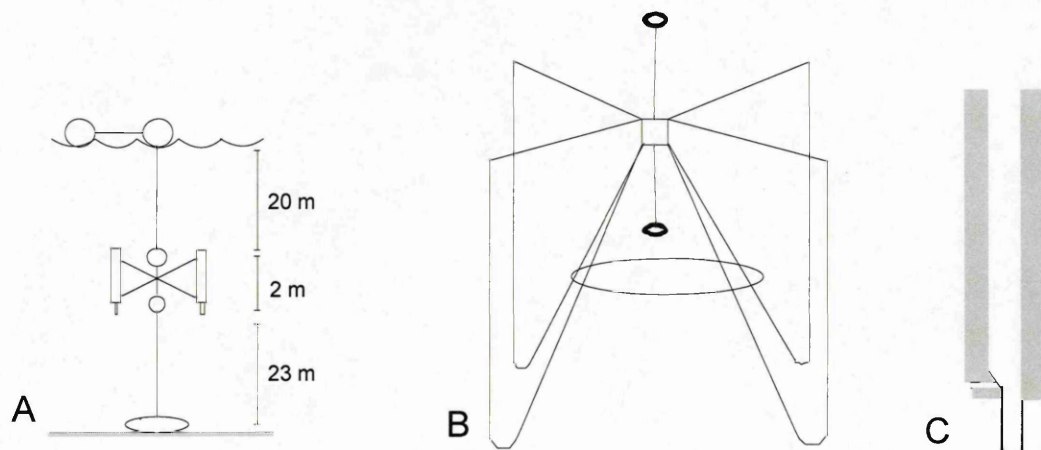
Air temperature and solar irradiance measurements were obtained from the meteorological station (HOBO Micro-Station Logger (H21-002) made by Onset Computer Corporation). The Micro-Station is fitted with two sensors; a solar radiation sensor (HOBO Silicon Pyranometer Smart Sensor) and a solar shielded air temperature sensor. The Micro-Station was installed at Dunstaffnage Marine Laboratory in 2004, and the data is provided courtesy of Dr. Michael Burrows.



**Figure 5.1** Location of study site (Station 3) in Loch Creran, west coast of Scotland and Stations 1 to 8 along the Loch Creran-Loch Linnhe transect.

### 5.2.3 Sedimenting material

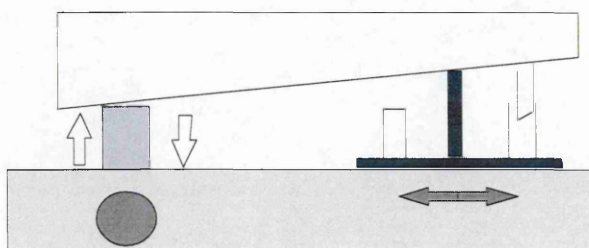
To collect sedimenting material, a sediment trap was suspended in the water column. The trap was constructed to the DML design (Leftley and MacDougall, 1991) and to the recommendations of Wassmann and Heiskanen (1988). It is composed of four collection cylinders held on a gimballed stainless steel frame (Fig. 5.2). This arrangement allows  $360^\circ$  rotation in the horizontal plane and about  $27^\circ$  tilt of the central rod in the vertical plane. Subsurface floats were secured above the frame. The internal diameter of a collection cylinder is 110 mm giving a collection area of  $0.0095 \text{ m}^2$  per cylinder and  $0.038 \text{ m}^2$  for the four cylinders. The traps were deployed from the *RV Seol Mara* using the winch system. The traps were moored at 20 m beneath the surface and 23 m from the bottom (Fig. 5.2). The traps were deployed for 24 hours with no fixative solution.



**Figure 5.2** Schematic diagram of a. of the position of the sediment trap in the water column, b. the sediment trap gimballed frame and c. a collection cylinder which is mounted onto the frame.

Following the recovery of the sediment trap equipment, the sediment trap material collected in the four receivers was immediately pooled on deck into a 500 cm<sup>3</sup> glass vial. During this procedure the sample material was siphoned through a 500 µm mesh to remove any large debris and swimming zooplankton that had been caught in the trap. The samples were stored in the dark and at ambient water temperature during transport to the laboratory for immediate analysis.

In the laboratory, the collected material was aliquoted for the analysis of biogenic sulphur and particulate carbon. Two methods were available for aliquoting: resuspension of material by gentle mixing and subsequent aliquoting manually or using a Sample Divider (Fig. 5.3). The Sample Divider has a reservoir (approximately 500 cm<sup>3</sup>) which drains into a rotating carousel of 10 x 20 cm<sup>3</sup> vials. A preliminary comparison of these two methods was made to investigate any detrimental effect on DMS levels and after the analysis of results, the Sample Divider was always used for aliquoting.



**Figure 5.3** Schematic diagram of the Sample Divider, used to aliquot sample material collected in the sediment trap.

Sample vials were analysed for DMS, DMSPp, DMSPd, DMSOp and DMSOd (in triplicate) by gas chromatography as detailed in Section 2.4. Analysis of particulate carbon was carried out in duplicate by filtering 5 cm<sup>3</sup> onto a glass fibre filter (Whatman

GF/F, 13 mm diameter) and a repeat of the analytical procedure as described in Section 5.2.2.

On three dates, the 18<sup>th</sup> March, the 19<sup>th</sup> April and the 27<sup>th</sup> June, sample material from the sediment trap was incubated over a 48 hour period to examine temporal changes in DMSP, DMSO and DMS concentrations. To obtain sufficient sedimenting material for analysis, an additional sediment trap of replicate design was deployed adjacent to the first trap and material collected by the same procedure. The combined sample was aliquoted using the Sample Divider to provide 20 replicate samples for incubation. The remaining samples were added to 20 cm<sup>3</sup> crimp top vials with no headspace. Three samples were immediately analysed to represent Time<sub>zero</sub> and the remaining vials were incubated in the dark at 10 °C. Samples were analysed for biogenic sulphur (in triplicate) at selected time intervals during a maximum incubation time of 55 hours.



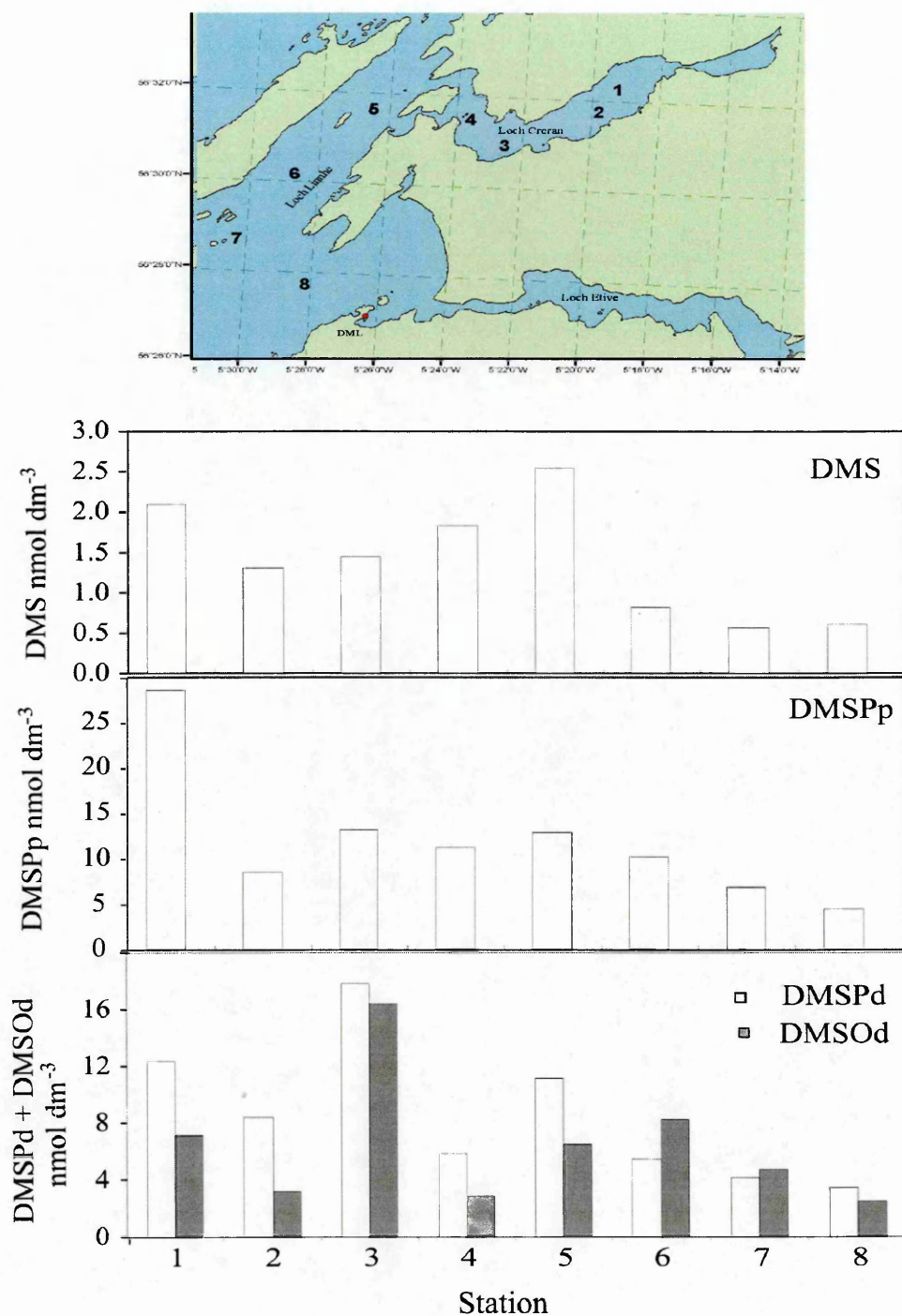
## 5.3 Results

### 5.3.1 Spatial distribution of DMS, DMSP and DMSO

The concentrations of biogenic sulphur compounds in the water column at the sample site were compared to other areas in Loch Creran and Loch Linnhe by conducting a transect on the 24<sup>th</sup> May 2005 (Table 5.2; Fig. 5.4). Along the transect DMS concentrations ranged between 0.6 nmol dm<sup>-3</sup> (Stn 7 & 8) and 2.5 nmol dm<sup>-3</sup> (Stn 5) with an overall average of 1.4 nmol DMS dm<sup>-3</sup> (Fig. 5.4). DMSPp concentrations displayed a general decrease seaward from an initial concentration of 28.5 nmol dm<sup>-3</sup> to 4.5 nmol dm<sup>-3</sup> at Stn 8 (Fig. 5.4). The exception was Station 2 which had the lowest concentration of DMSPp recorded along the transect route in Loch Creran of 8.6 nmol dm<sup>-3</sup>. The dissolved concentrations of DMSPd and DMSOd ranged from 3.5 to 17.8 and from 2.5 to 16.4 nmol dm<sup>-3</sup> respectively. Along the transect section within Loch Linnhe, DMSPd and DMSOd show a general decrease seaward, whilst within Loch Creran there is considerable variation associated with each station (Fig. 5.4). Overall, Loch Creran contained the highest concentrations for DMSPp, DMSPd and DMSOd, whilst the highest DMS concentrations were found at the mouth of the sea-loch. Station 3 was the site for the fortnightly sampling during March to June 2005.

Table 5.2 Grid reference of the transect stations, conducted on the 24<sup>th</sup> May 2005 together with the depth, temperature and salinity at each location.

Station	Grid reference		Depth (m)	Temp (°C)	Salinity (PSU)
	Latitude	Longitude			
1	56° 32.860N	05° 18.265W	24.0	10.5	29.1
2	56° 31.981N	05° 19.738W	23.6	10.5	25.8
3	56° 31.110N	05° 22.409W	38.0	10.5	31.1
4	56° 31.832N	05° 23.831W	13.8	11.0	30.3
5	56° 31.879N	05° 26.124W	32.2	10.0	31.0
6	56° 30.248N	05° 28.739W	50.9	11.5	28.6
7	56° 28.869N	05° 30.133W	47.2	11.5	26.6
8	56° 27.972N	05° 28.008W	40.3	11.0	28.1

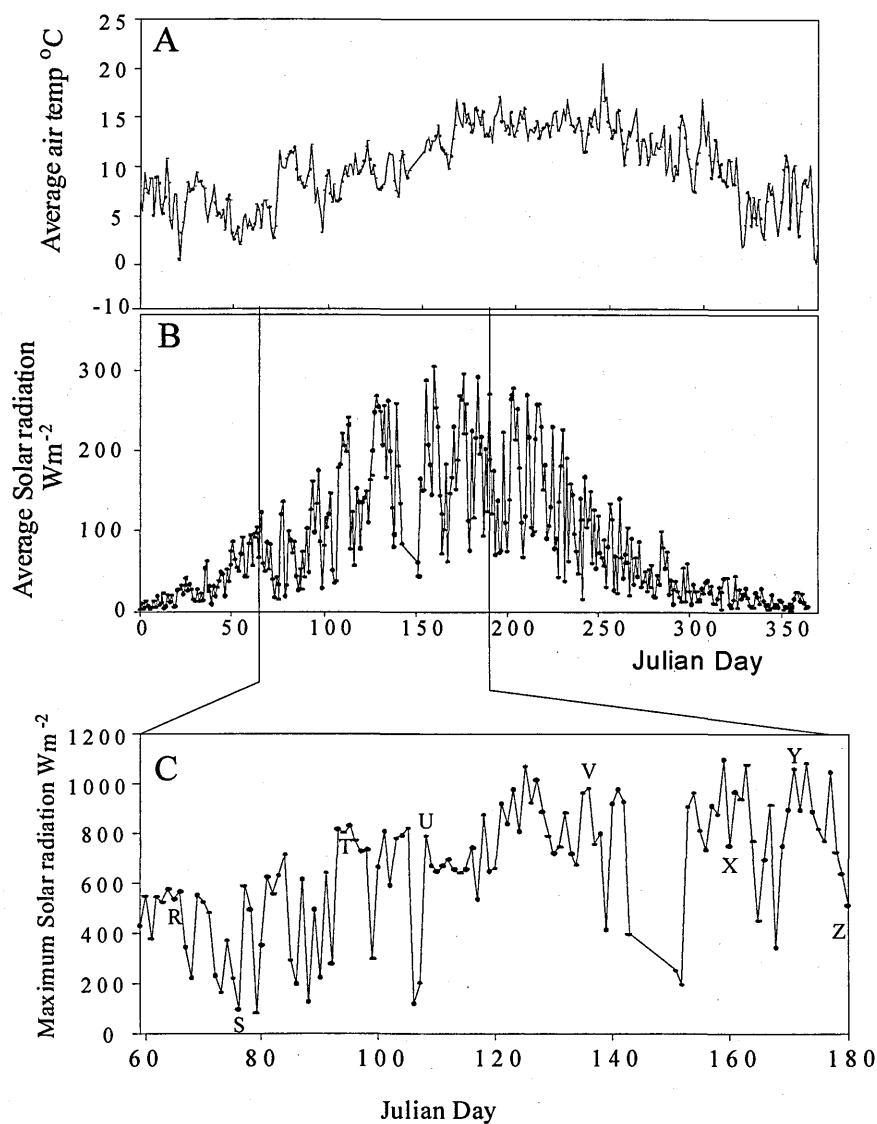


**Figure 5.4** Variation of DMS, DMSPp, DMSPd and DMSOd along a transect in Loch Creran and Loch Linnhe. The map of the transect stations is reproduced above (see Fig. 5.1 for larger version).

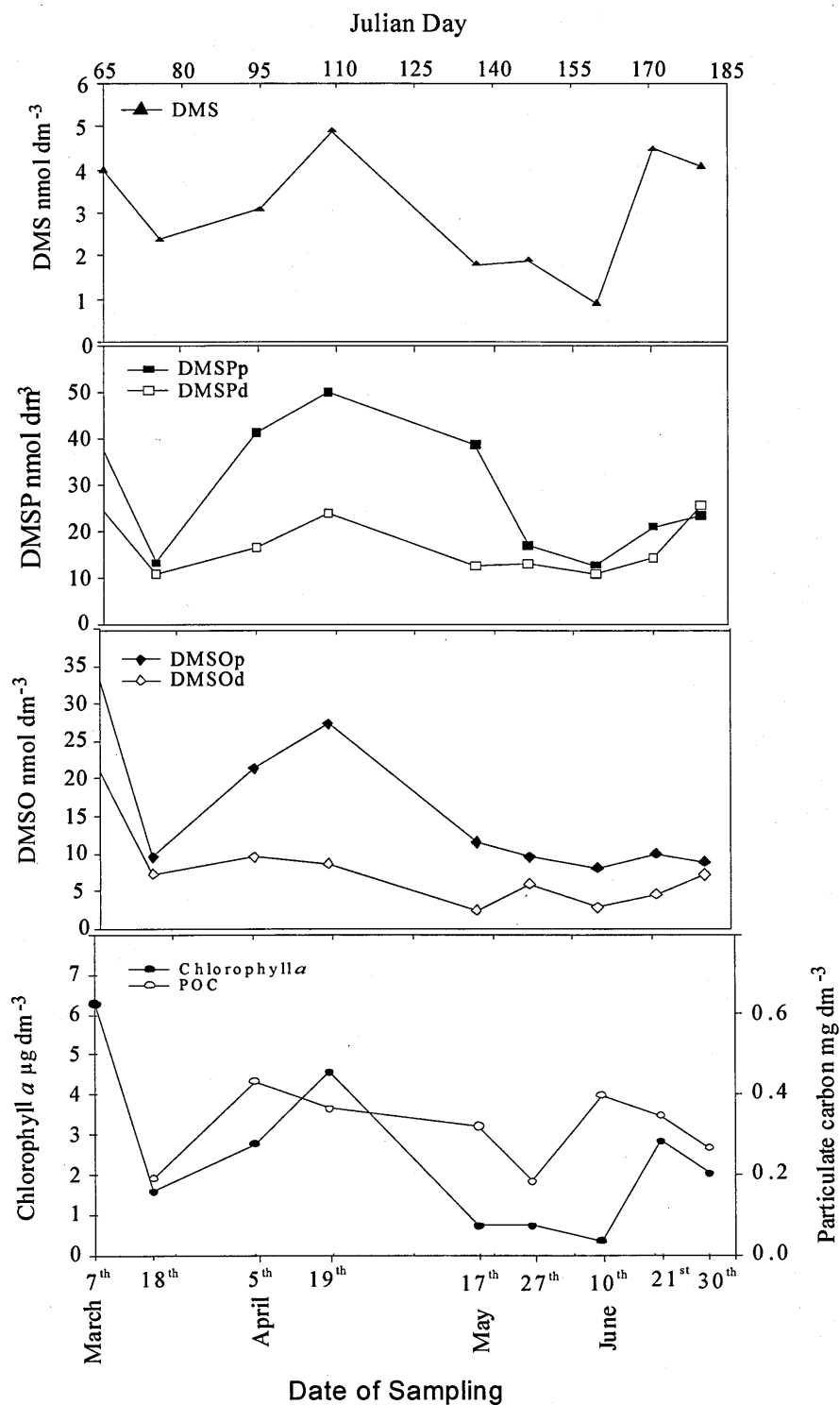
### 5.3.2 Temporal analysis of DMS, DMSP and DMSO in Loch Creran

Measurements of air temperature and solar irradiance were taken every 10 min by the Micro-Station Logger and averaged over the 24 hour period for 2005 (Fig. 5.5). The average air temperature over 24 hours during the sampling period (*i.e.* March to June 2005) was 9.7 °C, and an average solar irradiance over 24 hour period was 132.8 Wm<sup>-2</sup>. The maximum solar irradiance experienced per 24 hour period is also shown for the sampling period *i.e.* 1<sup>st</sup> March to June 30<sup>th</sup> (Fig. 5.5). An average for the maximum solar irradiance was 665 Wm<sup>-2</sup>, whilst May recorded the highest maximum solar irradiance of 824 Wm<sup>-2</sup> for all of the months during which sampling occurred.

During March to June 2005 at Stn 3, DMS concentrations ranged from 1 to 5 nmol dm<sup>-3</sup> with an overall average during this period of 3.1 nmol dm<sup>-3</sup> (Fig. 5.6). There are three occasions when higher levels of DMS are observed: 7<sup>th</sup> March; 19<sup>th</sup> April and the 21<sup>st</sup> June, corresponding to 4, 4.9 and 4.5 nmol dm<sup>-3</sup> respectively. DMSPp concentrations ranged from 12.7 to 50.1 nmol dm<sup>-3</sup> with an overall average of 28.5 nmol dm<sup>-3</sup> (Fig. 5.6). The periods of high DMSPp concentrations coincided with elevated concentrations of DMS. On these occasions, DMSPp increased to concentrations of 38, 50 and 28 nmol dm<sup>-3</sup> respectively. The overall average DMSPd concentration was 17 nmol dm<sup>-3</sup> and increased to 25, 23 and 25 nmol dm<sup>-3</sup> on the 7<sup>th</sup> March; 19<sup>th</sup> April and the 21<sup>st</sup> June respectively (Fig. 5.6). DMSOp concentrations ranged from 8.1 to 35.2 nmol dm<sup>-3</sup> with an overall average of 15.7 nmol dm<sup>-3</sup>. In general, DMSOp concentrations displayed a similar trend to DMSPp, with higher concentrations on the 1<sup>st</sup> March and also on the 19<sup>th</sup> April. DMSOd concentrations ranged from 2.4 to 22.3 nmol dm<sup>-3</sup> with an overall average of 7.9 nmol dm<sup>-3</sup>.



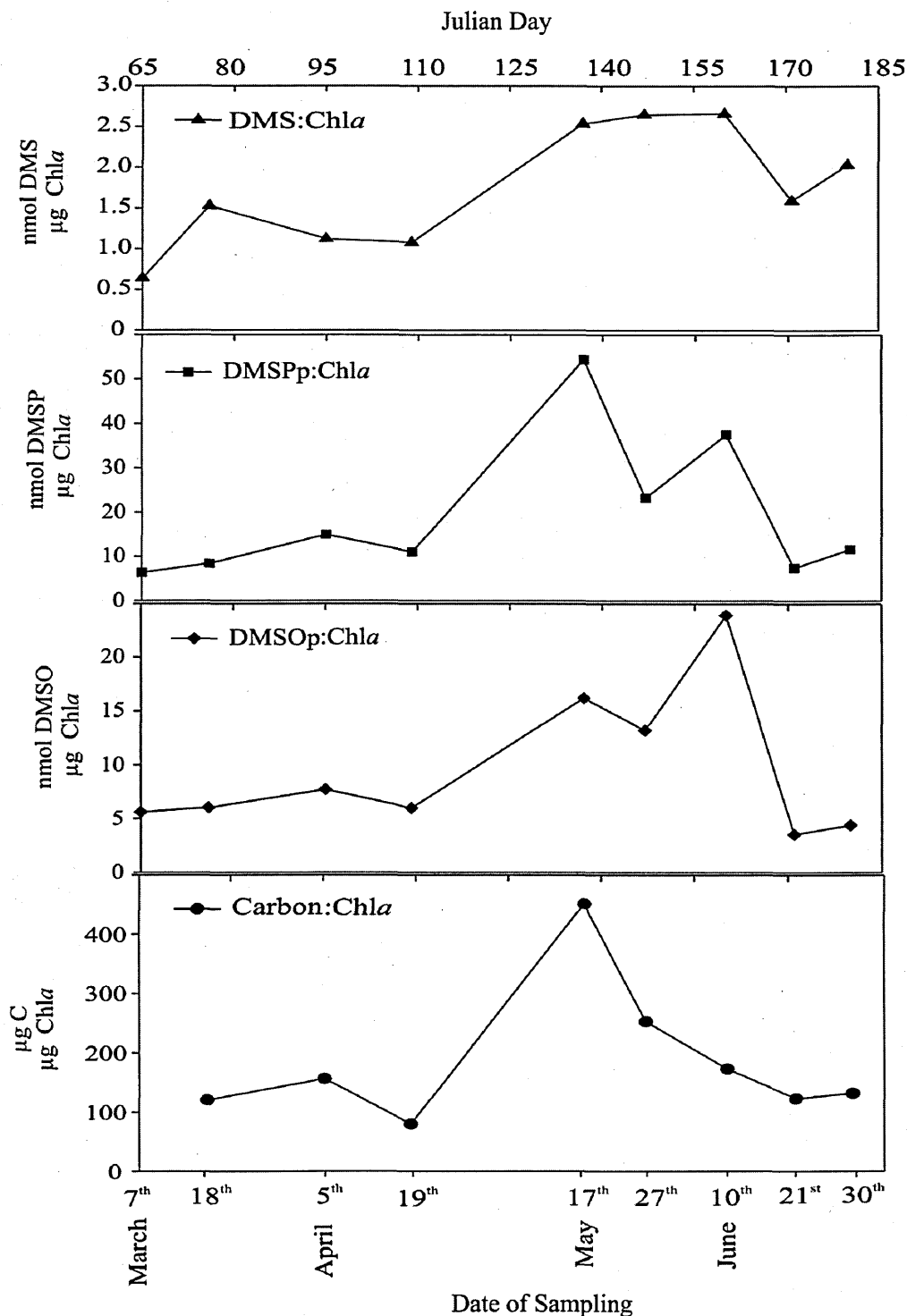
**Figure 5.5** Average air temperature (a) and solar irradiance (b) recorded over 24 hour period during 2005, and the maximum solar irradiance recorded over a 24 hour period for the sampling period (c). The letters on Fig 5.5c refer to sampling dates: R: 7<sup>th</sup> March; S: 18<sup>th</sup> March; T: 5<sup>th</sup> April; U: 19<sup>th</sup> April; V: 17<sup>th</sup> May; X: 10<sup>th</sup> June; Y: 21<sup>st</sup> June; and Z: 30<sup>th</sup> June.



**Figure 5.6** Concentrations of dimethylated sulphur compounds (DMS, DMSPp, DMSPd, DMSOp, DMSOd), POC and chlorophyll *a* during March to June 2005 at Station 3 in Loch Creran. The x-axis is provided in Julian Day at the top for comparison with Figure 5.5 and also by sampling date at the bottom for reference to the wider text.

The sampling period was characterised by average chlorophyll *a* concentrations of  $2.4 \mu\text{g Chl } a \text{ dm}^{-3}$  (Fig. 5.6). The three periods of elevated DMS and DMSPp concentrations are associated with elevated chlorophyll *a* concentrations and the 7<sup>th</sup> March, 19<sup>th</sup> April and the 30<sup>th</sup> June, corresponding to concentrations of  $5.78$ ,  $5.54$  and  $2.82 \mu\text{g Chl } a \text{ dm}^{-3}$  respectively. Carbon concentrations range from  $0.18$  to  $0.43 \text{ mg dm}^{-3}$ . The contribution of DMSP-carbon to the overall particulate carbon concentration in the upper 20 m of the water column was estimated *as per* Simo et al. (2000), by considering that 1 mole of DMSP contains 5 moles of carbon. Therefore, in this study, DMSPp represents 0.7 % of the particulate carbon budget (Table 5.4). In comparison, DMSOp (containing 2 moles of carbon) represents 0.28 % of the particulate carbon budget (Table 5.4).

Concentrations of DMS, DMSPp, DMSOp and particulate organic carbon (POC) are also shown normalised to Chlorophyll *a* (Fig. 5.7). The ratio of DMS:Chl *a* ranges from  $0.6$  to  $2.6 \text{ nmol } \mu\text{g dm}^{-3}$ . The DMSPp:Chl *a* ratio reveals low variation over the initial four sampling points with an average of  $10.7 \text{ nmol } \mu\text{g dm}^{-3}$ . On the 17<sup>th</sup> May, DMSPp:Chl *a* ratio increases to  $54 \text{ nmol } \mu\text{g dm}^{-3}$ , after which period a subsequent decrease is observed. DMSOp:Chl *a* steadily increase throughout the sampling period to reach a maximum concentration on the 10<sup>th</sup> June of  $23.9 \text{ nmol } \mu\text{g dm}^{-3}$ . Neither of the maximum concentrations in DMSPp:Chl *a* and DMSOp:Chl *a* coincide with the same dates when increased concentrations in bulk DMSPp and DMSOp were observed. (Fig. 5.7). Average POC:Chl *a* are  $186 \mu\text{g}:\mu\text{g dm}^{-3}$  and reach a maximum value on the 21<sup>st</sup> June of  $451 \mu\text{g}:\mu\text{g dm}^{-3}$ .



**Figure 5.7** Chlorophyll *a* normalised concentrations of dimethylated sulphur compounds (DMS, DMSPP and DMSOP) and POC during Spring 2005 in Loch Creran. The x-axis is provided in Julian Day at the top for comparison with Figure 5.5 and also by sampling date at the bottom for reference to the wider text.

### 5.3.3 The downward flux of DMSP and DMSO

A preliminary comparison of the two possible methods for sub-dividing the sample material collected in the sediment trap was initially performed. A one-way ANOVA showed that there was no significant difference between the Sample Divider and manually aliquoting (DMS:  $F_{1,7}=2.45$ ,  $P=0.161$ ; DMSPp:  $F_{1,10}=1.06$ ,  $P=0.319$ ). Importantly the Sample Divider was not found to affect the concentration of DMS through excessive turbulence. Subsequently, all sampling with the sediment trap used the Sample Divider to aliquot the material as it provided up to 20 replicates with a high degree of precision.

**Table 5.3** Comparison of DMS, DMSPp and DMSPd concentrations in particulate material when aliquoting the samples using a Sample divider and manually. The two rows, a and b, refer to a repeat of the experiment.

Method		DMS nmol dm <sup>-3</sup>	DMSPp nmol dm <sup>-3</sup>	DMSPd nmol dm <sup>-3</sup>
Sample Divider	a.	1.99 ± 0.1	163.9 ± 38.2	11.5 ± 1.3
	b.	5.27 ± 0.8	214.3 ± 36.1	15.8 ± 2.9
Manually aliquoting	a.	1.97 ± 0.1	149.8 ± 46.7	10.7 ± 1.4
	b.	4.56 ± 0.5	232.5 ± 3.91	15.9 ± 2.4

The material collected in the sediment trap was analysed for DMS, DMSP, DMSO and POC content. The sediment trap collecting area was then used to calculate the quantity of DMSPp or DMSOp that would have been collected over 1 m<sup>2</sup> and the daily flux rates were calculated by taking into account the duration of time the traps were deployed in the water column. The downward flux of DMSPp ranged from 19.4 to 84.1 µmol m<sup>-2</sup> d<sup>-1</sup> and the downward flux of DMSOp ranged from 7.03 to 21.97 µmol m<sup>-2</sup> d<sup>-1</sup> (Table 5.4). The importance of sedimentation as a sink for DMSPp and DMSOp can be assessed by comparing their downward flux with the integrated standing stock of the respective



compound in the waters above the sediment trap *i.e.* the top 20 m of the water column.

The integrated stock should be treated with caution as it represents two sampling points, the surface and 20 m depth. Results show that the DMSPp sedimentation flux ranged between 2.6 and 20 % of the DMSPp standing stock. The sedimentation of DMSOp accounted for 7.03 to 21.97  $\mu\text{mol m}^{-2} \text{d}^{-1}$  of the DMSOp standing stock.

**Table 5.4** The integrated standing stock, the downward flux, and the accumulation rates of A) DMSPp; B) DMSOp; and C) POC in the top 20 m of the water column.

<b>A.</b>	<b>Integrated standing stock DMSPp <math>\mu\text{mol m}^{-2}</math></b>	<b>% contribution of DMSP-carbon to POC</b>	<b>Daily flux DMSPp <math>\mu\text{mol m}^{-2} \text{d}^{-1}</math></b>	<b>Sedimentation rate <math>\% \text{d}^{-1}</math></b>
<b>Date</b>				
7 <sup>th</sup> March	917.5	-	71.3	7.8
18 <sup>th</sup> March	285.0	2.22	56.9	20.0
5 <sup>th</sup> April	748.8	3.05	19.4	2.6
19 <sup>th</sup> April	885.0	4.41	27.6	3.1
17 <sup>th</sup> May	776.3	3.86	84.1	10.8
27 <sup>th</sup> May	347.5	2.95	47.7	13.7
10 <sup>th</sup> June	287.5	1.02	23.8	8.3
21 <sup>st</sup> June	417.5	1.93	37.6	9.0
30 <sup>th</sup> June	530.0	2.82	27.9	5.3
<b>Average</b>	<b>577.2</b>	<b>2.8</b>	<b>44.0</b>	<b>9.0</b>
<b>SD</b>	<b>257.2</b>	<b>1.1</b>	<b>22.7</b>	<b>5.5</b>

<b>B.</b>	<b>Integrated standing stock DMSOp <math>\mu\text{mol m}^{-2}</math></b>	<b>% contribution of DMSO-carbon to POC</b>	<b>Daily flux DMSOp <math>\mu\text{mol m}^{-2} \text{d}^{-1}</math></b>	<b>Sedimentation rate <math>\% \text{d}^{-1}</math></b>
<b>Date</b>				
7 <sup>th</sup> March	828.8	-	16.95	2.04
18 <sup>th</sup> March	228.8	0.32	13.58	5.94
5 <sup>th</sup> April	332.5	0.32	14.82	4.46
19 <sup>th</sup> April	425.0	0.48	11.00	2.59
17 <sup>th</sup> May	348.8	0.23	21.97	6.30
27 <sup>th</sup> May	236.3	0.34	13.00	5.50
10 <sup>th</sup> June	168.8	0.13	7.03	4.16
21 <sup>st</sup> June	193.8	0.18	9.34	4.82
30 <sup>th</sup> June	197.5	0.21	14.71	7.45
<b>Average</b>	<b>328.9</b>	<b>0.28</b>	<b>13.6</b>	<b>4.8</b>
<b>SD</b>	<b>205.9</b>	<b>0.1</b>	<b>4.4</b>	<b>1.7</b>

Table 5.4 continued

<b>C. Date</b>	<b>Integrated standing stock particulate carbon mg m<sup>-2</sup></b>	<b>Daily flux Particulate carbon mg m<sup>-2</sup> d<sup>-1</sup></b>	<b>Sedimentation rate % d<sup>-1</sup></b>
18 <sup>th</sup> March	3500.0	-	-
5 <sup>th</sup> April	8285.7	98.2	1.19
19 <sup>th</sup> April	5970.8	36.4	0.61
17 <sup>th</sup> April	7000.1	-	-
17 <sup>th</sup> May	4330.4	117.9	2.72
10 <sup>th</sup> June	8065.5	133.1	1.65
21 <sup>st</sup> June	7657.7	140.4	1.83
30 <sup>th</sup> June	5434.5	71.3	1.31
<b>Average</b>	<b>6280.6</b>	<b>99.6</b>	<b>1.6</b>
<b>SD</b>	<b>1771.5</b>	<b>39.8</b>	<b>0.7</b>

#### 5.3.4 Variation in DMSP and DMSO during sedimentation.

An initial indication of the changes in DMSPp and DMSOp with depth is shown by the changing variations in DMSPp:POC, DMSOp:POC, and DMSPp:DMSOp ratios at different depths in the water column and material collected in the sediment trap deployed in the water column for 24 hours (Table 5.5). DMSPp:POC ratios at the surface range from 32 to 138 nmol:mg dm<sup>-3</sup> with an average of 87 nmol:mg dm<sup>-3</sup>. This decreases to an average of 52.2 nmol:mg dm<sup>-3</sup> at 20 m and further declines to 3.7 nmol:mg dm<sup>-3</sup> in sediment trap material. DMSOp:POC follows a similar trend, with values at the surface ranging from 20.4 to 75 nmol:mg dm<sup>-3</sup> with an average of 43.2 nmol:mg dm<sup>-3</sup>, decreasing to an average of 29.4 nmol:mg dm<sup>-3</sup> at 20 m and further declines to 1.5 nmol:mg dm<sup>-3</sup> in sediment trap material. DMSPp:DMSOp show no significant change with depth for the whole sampling period, with an average of 2.1 nmol:nmol dm<sup>-3</sup>. A slight increase in material collected in the sediment trap with an overall average of 3.1 nmol:nmol dm<sup>-3</sup>.

**Table 5.5** A) DMSPp:POC; B) DMSOp:POC; and C) DMSPp:DMSOp ratios at different depths in the water column and within particulate material collected in a sediment trap deployed at 20 m.

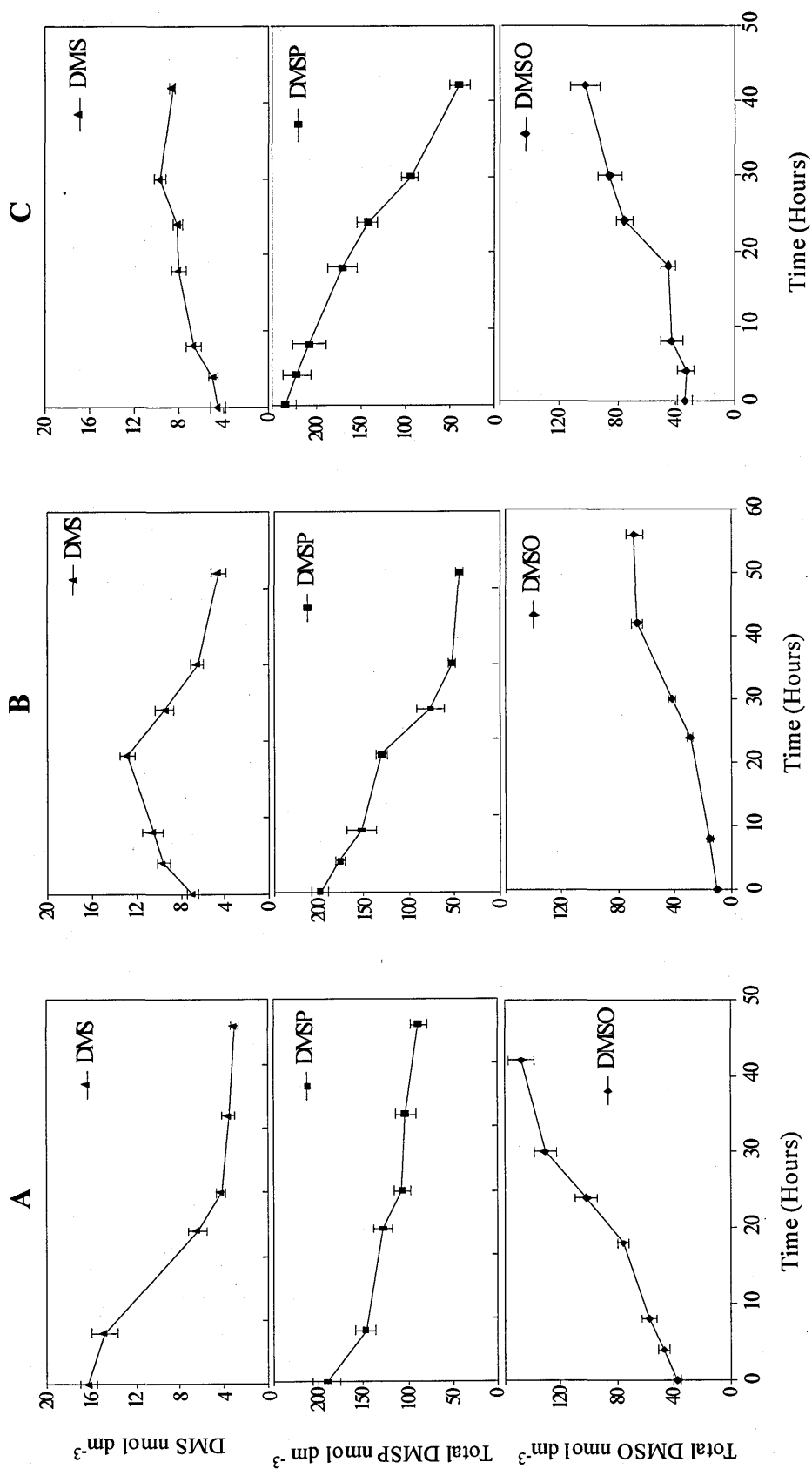
<b>A. DMSP: POC (nmol:mg dm<sup>-3</sup>)</b>			
<b>Date</b>	<b>Depth (Surface)</b>	<b>Depth (20 m)</b>	<b>Sediment trap material</b>
18 <sup>th</sup> March	69.5	50.5	-
5 <sup>th</sup> April	95.4	43.0	1.97
19 <sup>th</sup> April	137.9	57.0	7.58
17 <sup>th</sup> May	120.6	73.4	-
27 <sup>th</sup> May	92.0	61.2	4.05
10 <sup>th</sup> June	32.0	25.9	1.79
21 <sup>st</sup> June	60.5	35.7	2.68
30 <sup>th</sup> June	88.2	70.9	3.91
<b>Average</b>	<b>87.0</b>	<b>52.2</b>	<b>3.7</b>
<b>SD</b>	<b>31.3</b>	<b>15.6</b>	<b>2.0</b>

<b>B. DMSO:POC (nmol:mg dm<sup>-3</sup>)</b>			
<b>Date</b>	<b>Depth (Surface)</b>	<b>Depth (20 m)</b>	<b>Sediment trap material</b>
18 <sup>th</sup> March	50.0	46.3	-
5 <sup>th</sup> April	49.4	12.0	1.51
19 <sup>th</sup> April	75.1	18.4	3.02
17 <sup>th</sup> May	35.9	51.3	-
27 <sup>th</sup> May	52.4	51.8	1.10
10 <sup>th</sup> June	20.4	13.6	0.53
21 <sup>st</sup> June	28.8	15.8	0.67
30 <sup>th</sup> June	33.4	25.9	2.06
<b>Average</b>	<b>43.2</b>	<b>29.4</b>	<b>1.5</b>
<b>SD</b>	<b>16.1</b>	<b>16.2</b>	<b>0.9</b>

<b>C. DMSP:DMSO (nmol dm<sup>-3</sup>)</b>			
<b>Date</b>	<b>Depth (Surface)</b>	<b>Depth (20 m)</b>	<b>Sediment trap material</b>
18 <sup>th</sup> March	1.39	1.09	4.19
5 <sup>th</sup> April	1.93	3.58	1.31
19 <sup>th</sup> April	1.84	3.09	2.51
17 <sup>th</sup> May	3.36	1.43	3.83
27 <sup>th</sup> May	1.76	1.18	3.67
10 <sup>th</sup> June	1.57	1.91	3.39
21 <sup>st</sup> June	2.10	2.25	4.02
30 <sup>th</sup> June	2.64	2.74	1.89
<b>Average</b>	<b>2.1</b>	<b>2.2</b>	<b>3.1</b>
<b>SD</b>	<b>0.6</b>	<b>0.9</b>	<b>1.0</b>

### 5.3.5 Changes in DMS, DMSP and DMSO during incubation

The results for the three incubation experiments are shown in Figure 5.8. The results are presented as total DMSP and DMSO (*i.e.* particulate + dissolved fractions) to show the overall change in concentration for each of the compounds. The results clearly show a decrease in total DMSP concentration with time. In contrast, concentrations of total DMSO show an increase over the incubation period. Levels of DMS show an initial increase in two of the three experiments (Fig 5.8b, c) and subsequently decrease. The quantity of total DMSO produced can also be estimated as a fraction of the total DMSP lost, which ranges from 37 to 75 %.



**Figure 5.8** Changes in biogenic sulphur compounds in three incubation experiments conducted using material collected in a sediment trap deployed in a Scottish sea-loch, A: 18<sup>th</sup> March; B: 19<sup>th</sup> April; and C: 27<sup>th</sup> June. The results are the means for triplicate samples with standard error bars. Trap material was incubated in the dark and *in situ* water temperature on the three occasions. Graphs show changes in DMS, total DMSP (particulate + dissolved) and total DMSO (particulate + dissolved).

## 5.4 Discussion

### 5.4.1 DMSP, DMSO and DMS pools

The biogenic sulphur concentrations at Station 3 were compared with other nearby coastal locations by conducting a transect through Loch Creran and Loch Linnhe. Overall, average concentrations of DMS, DMSPp, DMSPd and DMSOd were higher in Loch Creran than Loch Linnhe. This is a likely consequence of the phytoplankton standing stock and community composition, which in turn is influenced in the coastal environment by nutrient concentrations, seaward drift and hydrological features such as the vertical water column structure (Tett and Wallis, 1978). Although the temperature profile was not recorded at each station, it is likely that the water column is more stable in Loch Creran which is conducive to a higher standing stock of phytoplankton (Tett and Wallis, 1978; Jones, 1979). In contrast, the stations within Loch Linnhe are more exposed to the open sea and are more susceptible to wind stress and fresh water input from Loch Etive and Loch Creran. A similar transect of biogenic sulphur compounds was also carried out in the Saguenay Fjord, Canada and included both the particulate and dissolved fractions of DMSO (Lee et al., 1999). Similar to Loch Creran, concentrations of DMS decreased seaward although by a much larger fraction, decreasing by a factor of 10, from 0.14 to 0.012 nmol dm<sup>-3</sup>. Previous work by Iverson et al. (1989) found that concentrations of DMS, DMSPd and DMSPp normalised to chlorophyll *a* increased from estuarine through to shelf and oceanic waters in three locations on the east coast of North America. This increase seawards correlated to algal community composition, with the estuarine diatom dominated communities producing less DMSP:Chl *a* than oceanic coccolithophore-dominated communities.

Between March to June 2005, DMS concentrations at Station 3 ranged from 0.9 to 4.9 nmol dm<sup>-3</sup>. These values are within the range reported for the nearshore waters around mainland Britain (0.3 to 34 nmol dm<sup>-3</sup>) (Turner et al., 1988), the southern North Sea (0.3 to 25 nmol dm<sup>-3</sup>) (Turner et al., 1996) and on the European western continental margin (3 to 20 nmol dm<sup>-3</sup>) (Uher et al., 2000). There is no evidence of DMS concentrations increasing as spring progresses as previously reported for this time of year in time-series measurements at other locations (Leck and Persson, 1996; Turner et al., 1996; Dacey et al., 1998). The concentrations of DMSPp in Loch Creran during the sampling period range from 12.7 to 50.1 nmol dm<sup>-3</sup>. These concentrations are at the lower end of DMSPp values observed in other coastal environments such as a Norwegian fjord (up to 170 nmol dm<sup>-3</sup>), the central Gulf of Lawrence (6-177 nmol dm<sup>-3</sup>) and coastal waters around the coast of Britain (14-288 nmol dm<sup>-3</sup>) (Turner et al., 1988). Concentrations of DMSOp in this study ranged from 8.1 to 27.3 nmol dm<sup>-3</sup> and are within the range reported by Simó and Vila-Costa (2006) for a wide variety of open ocean and marine sites of 1 to 40 nmol DMSOp dm<sup>-3</sup>. An overall average DMSPp:DMSOp ratio of 2 is calculated for the surface waters, which is lower than the overall average ratio of 4.85 reported for a range of laboratory cultures of phytoplankton in Chapter 3 respectively. This can be accounted for by the low DMSPp:DMSOp ratios associated with the diatoms *Chaetoceras debilis* and *Thalassiosira pseudonana*. The DMSPp:DMSOp ratios calculated for *C. debilis* and *T. pseudonana* in Chapter 3 were 1.14 and 1.93 respectively. Both of these species are typically found in Loch Creran (see below for discussion on algal community composition). Low DMSPp:DMSOp ratios have also been found elsewhere in the marine ecosystem, including the Arctic ecosystem (Lee et al., 1999; Bouillon et al., 2002) and the Peruvian Upwelling System (Riseman and DiTullio, 2004), as previously discussed in Chapter 3.

The dissolved concentrations of DMSPd and DMSOd show similar trends in the surface water. A high concentration is initially observed on the 7<sup>th</sup> March, followed by relatively low fluctuations in concentration, in comparison to DMSPp and DMSOp. The DMSPd levels (10.8–25.6 nmol dm<sup>-3</sup>) are also at the lower end of the range measured in British coastal waters (5.6–198 nmol dm<sup>-3</sup>) (Turner et al., 1988). A long-term time-series of biogenic sulphur at the Bermuda Atlantic Time Series, revealed DMSPd concentrations did not exhibit any real variation throughout the year, except on sporadic occasions during spring, which was in contrast to the considerable variations in DMSPp and DMS (Dacey et al., 1998). The dissolved pool of DMSP was recently reviewed by Kiene and Slezak (2006) who concluded that values of DMSPd were generally over-estimated due to the sampling methodology (as discussed in Chapter 3, Section 3.4). The authors concluded that a more realistic estimation of the global DMSPd value was <3 nmol dm<sup>-3</sup> compared to 16 nmol dm<sup>-3</sup> as previously reported by Kettle et al. (1999). The proposed re-estimated average value of DMSPd proposed by Kiene and Slezak (2006) was suggested to reflect the high affinity of bacteria for DMSPd (Kiene et al., 1998; Zubkov et al., 2002).

The contribution of DMSPp and DMSOp to the particulate carbon in the upper water column was calculated at 0.7 and 0.28 % respectively. This is within the range reported by Kiene et al. (2000) of 0.2 to 39 % for a range of coastal and open ocean sites. A general observation from the literature is that DMSPp as a percentage of total carbon is less than 1 % in estuarine or near-shore coastal waters which have a high organic loading (*e.g.* Chesapeake Bay: 0.2 % (Iverson et al., 1989); Duplin River: 0.07 to 0.8 % (Kiene et al., 2000); Loch Creran: 0.7 (this study)). In contrast, at open ocean sites the contribution of DMSP to the particulate carbon budget exceeds 1 % (*e.g.* sub-polar N Atlantic: 7 % (Simo et al., 2002), Atlantic Ocean: 1.7 % (Burgermeister et al., 1990); NE Pacific: 3 % (Bates et



al., 1994)). The fraction of particulate carbon that is found as DMSOp is not usually calculated in biogenic sulphur studies, however this study shows that it can represent almost half the contribution of DMSPp.

The ratio of DMSP:Chl *a* is used to estimate the proportion of algal DMSP-producers that exist in the natural phytoplankton assemblage. Therefore the highest DMSP:Chl *a* ratios are found in samples with high DMSP producers; *e.g.* dinoflagellates range from 33 to 124 nmol  $\mu\text{g}^{-1}$ ; prymnesiophytes range from 15 to 41 nmol  $\mu\text{g}^{-1}$ . Lower DMSP:Chl *a* ratios are associated with diatoms and typically range from 0.26 to 13.40 nmol  $\mu\text{g}^{-1}$  (Keller and Korjeff-Bellows, 1996). Unfortunately phytoplankton taxonomic composition measurements are not available to directly relate the algal species to the distribution of DMS, DMSPp or DMSOp in this study. However previous studies have reported on both the annual (Tett et al., 1981; Fehling, 2004) and seasonal cycle (Hatton, *unpublished data*) of phytoplankton in Loch Creran. Diatoms, predominantly *Skeletonema costatum*, also *Thalassiosira pseudonana*, *Pseudonitzschia* sp. and *Chaetoceras* sp. are often responsible for a spring bloom and for several subsequent peaks in chlorophyll *a*. Dinoflagellates are typically represented by *Scrippsiella trochoidea* and *Heterocapsa minuta*. Non-bloom conditions are characterised by a range of diatoms, dinoflagellates and other small flagellates. It is therefore considered likely that the low DMSPp:Chl *a*, observed from March to mid-April (Fig. 5.7) reflect diatom-dominated phytoplankton communities. The elevated DMSPp:Chl *a* concentrations in May (Fig. 5.7), possible reflect a dominance of the phytoplankton communities by dinoflagellates.

However the interpretation of DMSPp:Chl *a* ratios is rarely straightforward as in addition to the relationship between taxonomy and intracellular concentrations of DMSPp and

DMSOp, other factors such as photoadaptation (Belviso et al., 1993) and nutrient availability (Stefels and van Leeuwe, 1998) also influence the concentration of these compounds. Furthermore, intracellular DMSPp and DMSOp concentrations could respond differently to different cell stressors. This could explain why the highest concentrations in DMSPp:Chl *a* and DMSOp:Chl *a* ratios are observed at different times during this study. It is possible that some nutrients such as silicate, phosphate or nitrate were limiting, particularly towards the summer months, and this may have affect both the physiology and phytoplankton community composition (Fehling, 2004). During a laboratory culture study, Sunda (2002) postulated that phytoplankton increase intracellular DMS and DMSP relative to chlorophyll *a* levels under exposure to high UV radiation doses. A cellular antioxidant reaction was proposed whereby DMSP is degraded to DMS, which is subsequently oxidised to DMSO and methane sulfonic acid (Sunda et al., 2002). However a comparison between the dates when an increase in DMSP:Chl *a* and DMSO:Chl *a* was observed and the solar irradiance reveal no obvious abnormalities in the solar irradiance for these dates (Fig. 5.5).

The particulate organic carbon to chlorophyll ratio provides an indication of the variation in biomass to pigment values. A typical annual cycle in coastal waters would be a low ratio in spring (~20:1), corresponding to high chlorophyll *a* which would gradually decrease until mid-Summer when the POC:Chl *a* ratio could be expected to exceed 100:1 (Steele, 1962; Steele and Baird, 1965). The relatively high POC:Chl *a* ratios (400  $\mu\text{g}:\mu\text{g dm}^{-3}$ ) reported in this study for Loch Creran reflect the high particulate and detritus content of coastal waters and are within the range of values previously reported for these coastal waters by Ansell (1974).

### 5.4.2 Sedimentation of DMSP and DMSO

The downward flux of DMSPp in Loch Creran during the sampling period ranged from 19.4 to 84.1  $\mu\text{mol m}^{-2} \text{d}^{-1}$ . These sedimentation rates for coastal waters exceed the DMSPp flux measured during a coccolithophore bloom in the North Sea of 8.42 to 19.58  $\mu\text{mol DMSPp m}^{-2} \text{d}^{-1}$  (Hatton, 2002). The downwards flux of DMSOp ranged from 7 to 21.9  $\mu\text{mol m}^{-2} \text{d}^{-1}$  which also exceeds the measurements during the coccolithophore bloom of 0.58 to 2.34  $\mu\text{mol DMSOp m}^{-2} \text{d}^{-1}$  (Hatton, 2002). The increased sedimentation reflects the high turnover rates of phytoplankton communities in the coastal waters (Sharples and Tett, 1994). A consequence of the high turnover rates is that a greater proportion of biomass will survive mineralization during sedimentation in the shallow water environments. Therefore in coastal areas, DMSP concentrations in the sediment can exceed those in the overlying water column and be a source of DMS to the surrounding environment (Nedwell et al., 1994).

The sedimentation rate for particulate carbon in Loch Creran ranged from 36 to 133  $\text{mg C m}^{-2} \text{d}^{-1}$  during the sampling period. This is less than an average net sedimentation rate of 217  $\text{mg C m}^{-2} \text{d}^{-1}$  calculated by Tyler (1983) for Loch Creran, which was based on previous estimates of sedimentation in Loch Creran of 52-107  $\text{g C m}^{-2} \text{yr}^{-1}$  (Ansell 1974) and 37-97  $\text{g C m}^{-2} \text{yr}^{-1}$  (Blanchard 1979). The sedimentation rates for Loch Creran in this study are similar to other fjord-like coastal ecosystems, such as Pojo Bay in Finland where sedimentation was 39  $\text{mg C m}^{-2} \text{d}^{-1}$  (Heiskanen and Tallberg, 1999). The sedimentation of particulate carbon equates to a downwards flux of 0.7–2.6 %  $\text{d}^{-1}$  of the overall integrated particulate carbon standing stock.

One issue when assessing the particle flux is the sampling methodology. An alternative method is to measure the concentrations of various radionuclides which are scavenged by particles to then model the flux of other components of the material (Buesseler et al., 1992). Both methods have intrinsic uncertainties related to their sampling nature, (Buesseler et al., 2005). Methodological issues associated with sediment trap deployment are problems of hydrodynamics, the effect of swimming organisms and solubilisation of material and these issues would have been present during deployment in Loch Creran. However, in a relatively shallow coastal site, the problem of resuspension of sedimenting material (Gasith, 1975; Bloesch, 1994) represents a greater problem than issues associated with trap efficiency. Both Ansell (1974) and Blanchard (1979) acknowledged the issue of resuspension in their estimates of sedimentation in Loch Creran. In a fjord-like coastal ecosystem in Finland, <90 % of the sedimenting material collected in a sediment trap was derived from secondary sources due to resuspension (Heiskanen and Tallberg, 1999). However, in Heiskanen and Talberg (1999) study the sediment traps were moored at 15 m and 30 m in a water column with a total depth of 38 m. The effect of resuspension is more pronounced close to the sea bed (Steele and Baird, 1972) and in Loch Creran, the trap was positioned at 20 m in a 45 m water column and deployed for a 24 hour period, which would reduce the likelihood of sampling material which has undergone secondary sedimentation. Furthermore, a comparison of the microbial communities in Chapter 7 revealed the same species composition between faecal pellets samples and sediment trap material supporting the likelihood of limited resuspension.

### 5.4.3 Changes in biogenic sulphur during sedimentation

On all instances the DMSPp:POC ratio decreased with depth (*i.e.* less DMSP relative to carbon). This is also observed with the DMSOp: POC ratios on the majority of the sampling occasions. However the decrease in DMSOp:POC ratio was lower compared to DMSPp: POC and on one occasion (19<sup>th</sup> April) the ratio of DMSOp: POC increased. This indicates that with increasing depth DMSPp is consumed quicker than DMSOp. This observation is supported by microbial turnover rates which are quicker for DMSPp than for DMSOp (Simó et al., 2000). It also offers an explanation for why DMSOd has been observed at greater depths than DMS or DMSPd (Hatton et al., 1998; Bouillon et al., 2002).

It should be noted that the DMSPp:DMSOp ratios for the water column and the sediment trap material do not support the hypothesis that DMSPp is consumed more rapidly than DMSOp. There is no overall change in the DMSPp:DMSOp ratio with depth or after being caught in the trap (Table 5.5c). If DMSPp is more rapidly metabolised by bacteria this would be reflected by a decreasing DMSPp:DMSOp ratio. Such a decrease in the DMSPp:DMSOp ratio with depth was observed in Lake Bonney, Antarctica, and this was also attributed to the transformation of DMSP to DMSO, via DMS (Lee et al., 2004). The reason for the absence of any transformation in this study is not evident at present. It may be due to the low initial DMSPp:DMSOp ratios of 2 for the water column. To resolve this issue, a much greater resolution of the water column profile is required.

The incubation of particulate material retained within the sediment trap revealed an increase in total DMSO over a 48 hour period, as previously observed by Hatton (2002b)

and also tentatively observed in the copepod faecal pellets (Chapter 4). This indicates that in addition to the consumption of DMSOd, as observed during grazing by *T. longicornis* on *S. trochoidea* (Chapter 4), the net production of DMSO can also occur in the water column. The increase in DMSO is suggested to result from the microbial oxidation of DMS, which in turn is derived from DMSP degradation. The transformation of DMS to DMSO could either be DMS acting as an electron donor (an energy source) or the enzymatic conversion of DMS with no energetic benefit for the organism. The organisms considered responsible are known to include ammonia oxidisers (Juliette et al., 1993), methanotrophs (Fuse et al., 1998), algae (Fuse et al., 1995), some strains of phototrophs (Zeyer et al., 1987; Visscher and van Gemerden, 1991; Hanlon et al., 1994) and other methylotrophs (Vila-Costa et al., 2006).

However, it is not clear why a net production of DMSO should occur. The reduction of DMSO is a wide-spread phenomenon amongst bacterioplankton (Griebler and Slezak, 2001) and its reduction rate has been used as a measure of respiratory activity in bacterioplankton (Vila-Costa et al., 2006). Therefore why should DMSO accumulate in incubations of particulate material collected from the pelagic environment? One clue is revealed in the observation of high levels of DMSOd in the natural environment. In the eastern section of Lake Bonney, Antarctica concentrations of DMSOd reached 270 nmol dm<sup>-3</sup> at a depth of 20-25 m (Lee et al., 2004). In comparison, at the same depth, DMS concentrations were 68.6 nmol dm<sup>-3</sup>, DMSOp concentrations were 0.75 nmol dm<sup>-3</sup> and DMSPp concentrations were 2.3 nmol dm<sup>-3</sup>. Similar to this study, the source of DMSO was considered to be the oxidation of DMS, and the authors proceed to speculate that DMSOd accumulated in to a greater extent in the eastern part of the lake due to unfavourable thermodynamics, *i.e.* insufficient reducing conditions. The reduction

potential ( $E_h$ ) profiles for Lake Bonney are described in a separate study by Lee et al (2004a) and high DMSO concentrations are observed where conditions are less reducing, with a reduction potential of 200-230 mV.

During this study, the redox potential of the incubated samples was not monitored. It is anticipated that throughout the incubation period, oxygen concentrations would decrease. Assuming that the sample was saturated with oxygen at Time<sub>zero</sub> ( $265 \mu\text{mol O}_2 \text{ dm}^{-3}$ ) and a bacterial respiration rate of  $100 \text{ mmol O}_2 \text{ m}^{-3} \text{ d}^{-1}$  (Fig. 9.2, Chapter 9), then sub-oxic conditions would be occurring towards the end of the experiment. The onset of anoxia would have affected the obligately aerobic bacteria. It is also possible that the reduction of DMSO to DMS occurred during this study, only it is not apparent from the net concentrations of DMS due to a tight coupling between the production and consumption of DMS (Simó et al., 2000).

Although it has been shown that DMSO may be generated at the expense of DMSP and DMS, this pathway does not account for all of the DMSP lost in Figure 5.8. DMSP is rapidly turned over by marine bacteria such as the *Roseobacter* and SAR11 (Gonzalez et al. 1999). It is also possible that in addition to these marine bacteria more commonly identified in the marine pelagic environment, the DMSP is metabolised, either directly or indirectly, by methanogenic Archaea. The potential for DMSP metabolism by methanogenic Archaea is examined in Chapter 6.

#### 5.4.4 Conclusions

This study reports on the sedimentation of DMSP and DMSO within a Scottish sea-loch throughout Spring 2005. A higher sedimentation rate for DMSP and DMSO was found in Loch Creran than previously reported for continental shelf and open ocean environments (Table 5.1). The incubation of particulate material collected in the sediment trap highlighted that within the upper water column the net production of DMSO can occur, whilst during the same experiment, a net consumption of DMSP and DMS was observed. The production of DMSO within particulate material also contrasts with the decrease in DMSO:POC ratio observed with increasing depth in the water column. At the moment it is not apparent why an increase in DMSO is observed in samples obtained from the pelagic environment, compared to a decrease in the grazing experiments, particularly as the incubated pelagic samples appear to represent more favourable conditions for bacterial respiration of DMSO. It is possible that levels of DMSO are controlled by its availability as either microbial carbon source or an electron acceptor.



## Chapter 6. Methane production in the pelagic environment

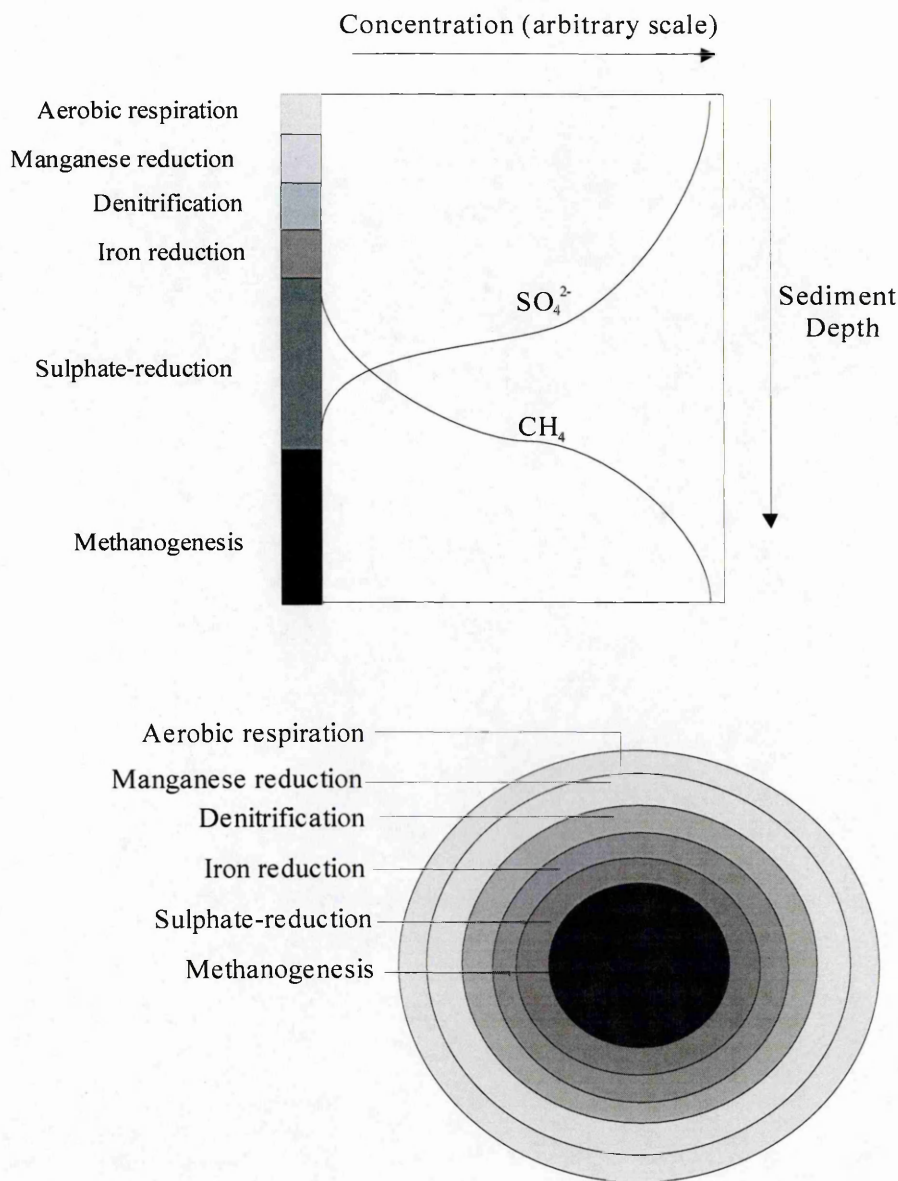
### 6.1 Introduction

Methane biogeochemistry in the upper water column reflects a combination of externally derived sources, oxidative processes, *in situ* production and the sea-air flux. The external sources have been described in Chapter 1 (Section 1.2.5) and include the microbially-mediated diagenesis of organic matter in the sediment, the abiotic production of methane and the decomposition of methane clathrate hydrates. These contributions of methane enter the oceanic water column via coastal run-off, by diffusion from organic-rich anoxic sediments and through seeps, vents and mud volcanoes emitting methane rich fluids or bubbles. These large additions of methane to the water column are effectively oxidised to low nanomolar levels so that most of the ocean volume is undersaturated with respect to the atmosphere (Reeburgh, 2007). Methane oxidation can occur under anaerobic conditions in marine sediments and under oxic conditions at the benthic boundary layer and in the water column. An additional sink for methane in the water column is the sea-air flux which has been estimated at 10-15 Tg CH<sub>4</sub> yr<sup>-1</sup> (IPCC, 2001). One of the most enigmatic processes of upper water column methane biochemistry, is the *in situ* production of methane in the surface mixed layer where concentrations of methane are frequently supersaturated with respect to the atmosphere (Scranton and Brewer, 1977). Because methane production occurs under strict anoxic conditions, the apparent production in oxic waters has been referred to as the 'ocean methane paradox' (as discussed in Section 1.2.5) (Kiene, 1991). Methanogenesis in the upper water column remains a poorly qualified process as the methanogen species responsible, the precise metabolic pathway and even the location of methane production remain open to debate.

To date, the majority of our current understanding of biological methane production and the other anaerobic respiration pathways is derived from sediment environments.

Sediments are reducing environments where aerobic micro-organisms are restricted to a thin oxygenated surface layer (Jorgensen, 1983). The stratification and immobile nature of sediments provides an insight into chemical and biological interactions that structure the anaerobic food web. Therefore in an idealised vertical profile of sediment, a gradient of aerobic-anaerobic respiratory pathways forms due to the energetics associated with each respiratory pathway and the availability of suitable terminal electron acceptors (Fig. 6.1).

One common feature of idealised vertical profiles of anaerobic sediments is an increase in methane concentrations when sulphate is sufficiently depleted (Martens and Berner, 1974) (Fig. 6.1). This is a consequence of sulphate-reducing bacteria (SRB's) out-competing methanogens when sulphate is freely available due to their higher affinity for the main substrates,  $H_2$  or acetate, used by both microbial groups (Kristjansson et al., 1982; Lovley et al., 1982). However, the competition between methanogens and SRB's does not explain how methane can be present in sulphate-rich environments *e.g.* salt-marsh sediments and other saline ecosystems (Oremland et al., 1982; King, 1984b). In these environments, methane is thought to result from methylotrophic methanogenesis, whereby the methyl group of a variety of  $C_1$ -compounds is reduced to methane (Fig. 1.9). Therefore the methanogens avoid competition with SRB's for the same electron donor, by metabolising methylated compounds (Oremland et al., 1982; King, 1984b).



**Figure 6.1a** An ‘idealized’ depth profile of methane and sulphate concentrations in marine sediments. The bar at the left-hand side represents the vertical distribution of respiration pathways. **Figure 6.1b** represents the idealized structure of an anaerobic microniche *e.g.* faecal pellet. Taken from Canfield et al. (2005).

Approximately 20 species of methanogens are known methylotrophs and phylogenetically they almost exclusively comprise the *Methanosarcinaceae* family (Keltjens and Vogels, 1993). The methylated compounds metabolised by methanogens are listed in Table 6.1. The first report on methane formation from a methylated C<sub>1</sub>-compound was based on methanol (Table 6.1, Equation 3) and since then it has been demonstrated that all methylotrophic methanogens are capable of deriving their energy from the conversion of methanol into CH<sub>4</sub> and CO<sub>2</sub> (Keltjens and Vogels, 1993). Subsequently, nitrogen-based methyl compounds were shown to be common substrates with the isolation of a methanogen strain that grew on mono-, di- and tri-methylamine (Hippe et al., 1979) (Table 6.1, Equations 4,5 & 6 respectively). The methylated sulphur compounds, DMS (Table 6.1, Equation 7), methanethiol (Table 6.1, Equation 8 & 9) and 3-methiolpropionate (Table 6.1, Equation 9), were relatively recently added to the list (Kiene et al., 1986; Oremland and Kiene, 1986). The metabolism of methyl-sulphur compounds by methanogens (as described in Chapter 1, Section 1.2.2) is likely to proceed along the same biochemical pathway as methanol and methylated amines, but involve distinct enzymatic processes (Lomans et al., 2002).

**Table 6.1** Sum reactions and energy yields reactions by methylotrophic methanogens, compared to  $H_2$  (Equation 1) and acetate (Equation 2). The references are: <sup>1</sup>Barker (1936); <sup>2</sup>Hippe (1979); <sup>3</sup>(Kiene 1986); <sup>4</sup>Finster (1992); <sup>5</sup>van der Maarel (1997).

	Reactants	Products	$\Delta G$ (kJ mol <sup>-1</sup> )	Reference
1	Hydrogen	$4 H_2 + CO_2 \rightarrow CH_4 + 2 H_2O$	-130.4	-
2	Acetate	$CH_3COO^- + H^+ \rightarrow CH_4 + 3 CO_2$	-211	-
3	Methanol	$4 CH_3OH \rightarrow 3 CH_4 + CO_2 + 2 H_2O$	-106	(1)
4	Mono-methylamine	$4 CH_3NH_2 + 2 H_2O \rightarrow 3 CH_4 + CO_2 + 4 NH_3$	-76.7	(2)
5	Di-methylamine	$2 (CH_3)_2NH + 2 H_2O \rightarrow 3 CH_4 + CO_2 + 2 NH_3$	-74.8	(2)
6	Tri-methylamine	$4 (CH_3)_3N + 6 H_2O \rightarrow 9 CH_4 + 3 CO_2 + 4 NH_3$	-74.8	(2)
7	Dimethylsulphide	$2 (CH_3)_2S + 2 H_2O \rightarrow 3 CH_4 + CO_2 + 2 H_2S$	-52.1	(3)
8	Methanethiol	$4 (CH_3)SH + 2 H_2O \rightarrow 3 CH_4 + CO_2 + 2 H_2S$	-51	(4)
9	Methanethiol	$(CH_3)SH + H_2 \rightarrow CH_4 + H_2S$	-69.3	(4)
10	3-Methiolpropionate	$4 CH_3SCH_2CH_2COO^- + 2 H_2O \rightarrow 3 CH_4 + HS-CH_2CH_2COO^- + CO_2$	-36	(5)

The metabolism of methylated sulphur compounds is not ubiquitous among methylophilic methanogens and only approximately half of the *Methanosarcinaceae* family demonstrate the potential to grow on methylated sulphur compounds (Table 6.2).

**Table 6.2** List of DMS-utilising methanogen species with place of origin (Updated from Lomans (2002)).

Name <sup>1</sup> (type strain)	Origin <sup>2</sup>	Substrate <sup>3</sup>	Reference
<i>Ms. acetivorans</i>	Scripps Canyon, California (M)	Me, Ma, MeS, Ac	Sowers (1994)
<i>Ms. barkeri</i>	-	Me, Ma, MeS	Tallant (1997)
<i>Ms. semesiae</i>	Mtoni Creek, Tanzania (MF)	Me, Ma, MeS	Lyimo (1998)
<i>Ms. siciliae</i>	Sicily Italy (M)	Me, Ma, MeS	Stetter (1989)
<i>Ms. siciliae</i>	Oil well, Mexico (M)	Me, Ma, MeS	Ni (1991)
<i>Ms. sp.</i>	Wadden Sea, Holland (M)	Me, Ma, MeS	van der Maarel (1997)
<i>Ml. bombayensis</i>	Arabian Sea, India (M)	Me, Ma, MeS	Kadam (1994)
<i>Ml. oregonensis</i>	Alkali Lake, Oregon (SL)	Me, Ma, MeS	Liu (1991)
<i>Ml. taylorii</i>	Francisco Bay, Oregon (E)	Me, Ma, MeS	Kiene (1986)
<i>Mh. zhilinae</i>	Bosa Lake, Egypt (SL)	Me, Ma, MeS	Mathrani (1989)
<i>Mm. hollandica</i>	Dekkerswald, Holland (F)	Me, Ma, MeS	Lomans (1999)
<i>Mm. hollandica</i>	Lake Baldegger (F)	Me, Ma, MeS	Simankova (2003)
<i>Strain MTP4</i>	Bordeaux, France (SM)	Me, Ma, MeS, Ac	Finster (1992)

<sup>1</sup> Abbreviation of taxa: *Ms.*: *Methanosarcina*; *Ml.*: *Methanobrevibacter*; *Mh.*: *Methanohalophilus*; *Mm.*: *Methanomethylovorans*.

<sup>2</sup> The origin of strain is coded by: SL : salt lake sediment; E: estuarine sediment; F: freshwater sediment; M: marine sediment; MF: mangrove forest sediment; SM: salt marsh sediment.

<sup>3</sup> The substrates for methanogens are: Me: methanol; Ma: methylamines; MeS: methylated sulphur compounds; Ac: acetate.

Within the marine environment, the presence of the biogenic sulphur compounds DMS, methanethiol and 3-methylthiopropionate is intimately linked with a precursory compound, dimethylsulphoniopropionate (DMSP). As observed in Chapter 3, DMSP occurs at high intracellular concentration in distinct groups of marine phytoplankton *e.g.* dinoflagellates and prymnesiophytes, where the main function appears to be as a compatible solute involved in osmoprotection. Much of our understanding of DMSP and its derivative compounds has resulted from a combination of research in both pelagic and benthic

ecosystems. For example, studies on DMSP degradation in anoxic coastal sediments revealed that anaerobic microbes can degrade DMSP via a demethylation pathway, without necessarily producing DMS (Kiene and Taylor, 1988). This involves an initial demethylation step to 3-methiolpropionate, followed by a subsequent demethylation process to produce 3-mercaptopropionate (Chapter 1; Fig. 1.4). This demethylation pathway was subsequently demonstrated for aerobic bacteria isolated from phytoplankton and coastal sediments (Taylor and Gilchrist, 1991). More recently, the demethylation pathway was demonstrated in isolates of *Roseobacter* (Gonzalez et al., 1999). This demethylation step appears to be a pivotal process in the microbial control hypothesis which suggests that the production of DMS is a consequence of microbial sulphur demand relative to ambient concentrations (Kiene et al., 2000).

It is apparent that the degradation of DMSP and its derivatives has been investigated for both anaerobic bacteria in the benthic environment and aerobic bacteria in the pelagic environment. However there are also indications that anaerobic bacteria can be metabolically active in the aerobic pelagic environment. The presence of the trace gases methane (Scranton and Brewer, 1977; Burke et al., 1983), sulphides (Cutter and Krahforst, 1988; Luther and Tsamakis, 1989), and hydrogen (Schropp et al., 1987b; Schropp et al., 1987a) in the upper ocean have been reported. Their presence has been used as evidence that the depletion of oxygen in micro-sites can occur (Sieburth, 1991). This is also the premise of Sieburth's (1991) 'micro-benthos' hypothesis: that anaerobic microbial activity typically associated with the benthic environment, can occur in micro-environments within the upper ocean (Fig 6.2). In support of this hypothesis, methanogens have been isolated from particulate material collected from the pelagic

environment (Cynar and Yayanos, 1991; Sieburth, 1993) as discussed further in Chapter 7.

Furthermore, all methanogenic Archaea identified from the upper ocean to date have been methylotrophic methanogens *i.e* derived from the family *Methanosarcinaceae*. This suggests that within the upper ocean methylotrophic methanogenesis may be the dominant metabolic pathway and not CO<sub>2</sub>-reduction or acetotrophy as reported for other anaerobic habitats (Meronigal et al., 2004). More specifically, it has been postulated that the methanogens isolated from the surface ocean are metabolising methylamines derived from phytoplankton (Cynar and Yayanos, 1991). Methylamines and glycine betaine are the nitrogen analogues of DMS and DMSP and are considered to have a similar osmotic function in marine phytoplankton (Keller et al., 1999a). The role of methylamines as precursory substrates for methanogens is well-established (King, 1984a), and the fermentation of glycine betaine to simple amines and subsequent utilization by methanogens has been observed in methanogen enrichments from hypersaline algal mats (King, 1988) and freshwater sediments (Hippe et al., 1979). In contrast to DMSP, glycine betaine appears to be a minor constituent of algal cells (Keller et al., 1999b). However although DMSP is present in higher concentrations within algal cells, it is not typically considered a metabolic substrate for methanogens in the upper water column and has never been specifically tested as a precursor for methanogenesis in sample material collected from the pelagic environment.

The aim of this study was to investigate the role of methylated sulphur compounds: DMSP, DMSO and DMS in the production of methane within sample material collected



from the upper water column. The work initially looked at copepod faecal pellets as a site for methanogenesis. Following on from this work, analysis for methane was performed on particulate material collected in sediment traps. Sample material was incubated with amendments consisting of different methylated sulphur compounds and inhibitors, to look at the stimulation and inhibition of methanogenesis. These incubation experiments were replicated with material collected from the benthic habitat to compare the microbial activity in the sediment environment and the upper water column.

## 6.2 Method

### 6.2.1 Methane production in copepod faecal pellets

Copepod faecal pellet samples were collected from *Temora longicornis* which was maintained in mono-culture, as described in Chapter 4. The copepods were kept at 14 °C and fed *Rhinomonas* sp. and the heterotrophic dinoflagellate *Oxyrrhis marina*. To collect faecal material, adult copepods were starved for 48 hours, fed and then left to defecate for 3 hours. Faecal pellets were collected on a 32 µm mesh, transferred to a glass Petri dish and examined under a light microscope for counting and to remove any non-faecal material. Approximately 600 faecal pellets (total sample volume ~0.2 mg (Chapter 7)) were transferred to 10 cm<sup>3</sup> glass vials (in triplicate). The sample was reduced by adding 100 µl of freshly prepared sodium dithionite (Sigma Aldrich) solution as a reducing agent to a final concentration of 200 µmol dm<sup>-3</sup>, as reported by Vester and Ingvorsen (1998). Within the sample vial, a 2 cm<sup>3</sup> headspace was created using oxygen-free nitrogen, and the sample was subsequently purged for 5 min. Every 2-3 days for a period of 2 weeks, the samples were analysed for headspace methane, using the analytical procedure as previously described (Chapter 2, Section 2.2). The experimental set-up was repeated with the inclusion of DMSP (final concentration of 20 nmol dm<sup>-3</sup>). To add the DMSP to the sample vial, a hyperdermic needle was used to inject the substrate through the rubber seal into the vial after it had been purged to become anaerobic. Control samples consisted of seawater, and seawater+DMSP and were established using the same procedure as above.

### 6.2.2 Methane production in pelagic and benthic material

To investigate methane production in sample material collected from the pelagic and benthic environment, sample material from these habitats was used to create anaerobic slurry microcosms. Slurry microcosms have been widely used to look at the response of microbial communities from a particular environment *e.g.* benthic environment (Purdy et al., 2003b; Purdy et al., 2003a), water samples (Massana et al., 2001), rice field soil (Chin et al., 1999). Experiments using slurry microcosms involve the collection of sample material and subsequent incubation under the appropriate conditions (*e.g.* anaerobic, elevated CO<sub>2</sub> conditions). The response of the microbial community throughout the incubation period can be monitored by analysing the metabolic by-products *e.g.* methane (Purdy et al., 2003b) or using molecular techniques, *e.g.* denaturing gradient gel electrophoresis (Massana et al., 2001).

#### *Pelagic material*

Sedimenting material was collected from the pelagic environment using the two sediment traps, as described in Chapter 5. The sediment trap was deployed on three occasions during summer 2006: May 20<sup>th</sup>; June 8<sup>th</sup>; and August 11<sup>th</sup>. On each sampling occasion the sediment traps were deployed for 4 days to obtain sufficient material for analysis. After retrieval of the sediment trap, sample material from the collecting tubes was pooled by siphoning through a 500 µm mesh to remove any large debris or zooplankton caught in the trap. Samples were returned to the laboratory on ice to prepare the slurry microcosms. In addition, seawater concentrations of methane within the sediment trap receiver were analysed. After retrieval of the sediment trap, seawater was carefully

decanted in to 60 cm<sup>3</sup> glass vials and crimp-sealed without headspace. Methane was analysed as detailed in Chapter 2; Section 2.2.

### *Benthic material*

Sediment was collected from the benthic environment on May 18<sup>th</sup> and August 20<sup>th</sup> from two sites within Loch Creran (Fig. 6.2). On each occasion, benthic material was collected using different sampling techniques due to logistical reasons. For May 18<sup>th</sup>, the SAMS multiple corer (Bowers & Connelly) was deployed which retrieves virtually undisturbed benthic samples using a series of 10 cm diameter cores (Barnett et al., 1984). For August 20<sup>th</sup> sample, a box core was deployed. On both occasions, sub-samples of sediment were collected from a 5-8 cm depth horizon for slurry experiments by using a 20 cm-long minicore. This consisted of a 20 cm<sup>3</sup> syringe with the Luer end removed.

The sample material from benthic and pelagic environment were analysed for background levels of biogenic sulphur. For each sample 0.3 g (wet weight) of material was added to 10 cm<sup>3</sup> glass vial containing 1 cm<sup>3</sup> 10 M NaOH and 9 cm<sup>3</sup> de-ionised water. This was performed in triplicate and samples were analysed for Total DMS + DMSP, see Section 2.1.2. The wet/dry weight ratio of samples was quantified by oven-drying 0.5 g of sample material at 60 °C overnight.

### **6.2.4 Slurry incubations**

To create the slurry microcosms, 1 g (wet weight) triplicate aliquots of sample material were added to a sterile 20 cm<sup>3</sup> glass vial. The glass vials were filled with 0.5 µm filtered autoclaved seawater and sealed using aluminium crimp seals with butyl rubber lining. A

3 cm<sup>3</sup> headspace of oxygen-free nitrogen was introduced to the glass vial using a hyperdermic needle by expelling the seawater with a second needle. The slurry was subsequently purged with nitrogen for 5 min to remove all oxygen. The slurry microcosms were amended with selected substrates and specific inhibitors of key functional groups (see below) by injecting them through the crimp seal into the anaerobic media. Controls flasks were also established for each experiment. The controls consisted of a filtered seawater control; an autoclaved control (121 °C for 20 min); and also seawater with the relevant amendment (substrate or substrate+inhibitor).

The slurry microcosms were incubated in the dark at 13 °C and mixed by manual rotation twice daily. The headspace gas was analysed for methane every 2 days using FID gas chromatography, as previously described (Chapter 2, Section 2.2). Typically 2 cm<sup>3</sup> of headspace was removed by a gas tight 10 cm<sup>3</sup> syringe and injected into the GC preparatory system. This was replaced by 2 cm<sup>3</sup> of nitrogen.

#### *Amendments - Substrates*

To investigate the metabolism of methylated compounds, the substrates glycine betaine (GBT), dimethylsulphoniopropionate (DMSP), dimethylsulphide (DMS) and dimethylsulphoxide (DMSO) were added separately on different sampling occasions at a final concentration of 20 nmol dm<sup>-3</sup> (Table 6.3). These concentrations are similar to ambient concentrations of DMS, DMSP and DMSO previously measured in Loch Creran, as discussed in Chapter 5.

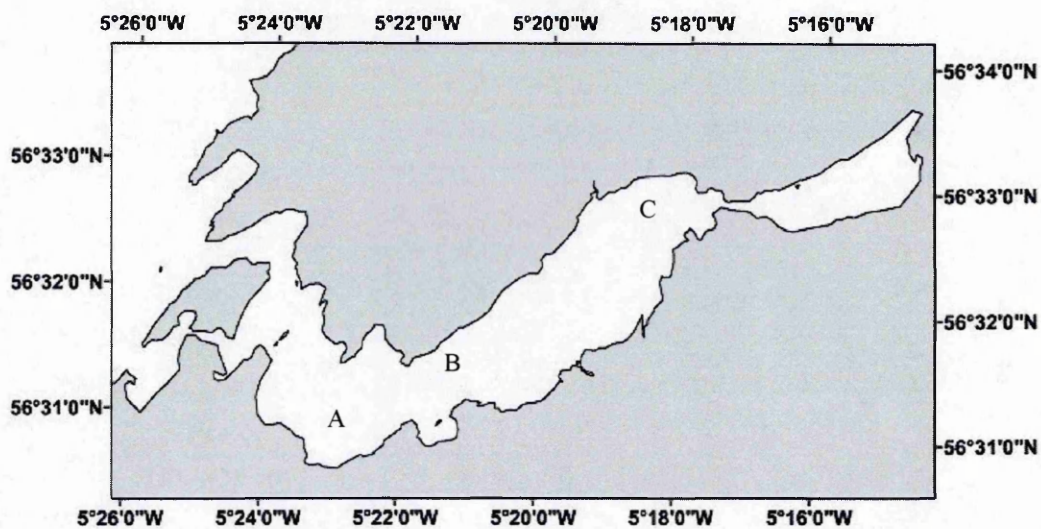
*Amendments – Inhibitors*

To investigate the activity of wider bacteria consortia, two inhibitors, molybdate and antibiotics (ampicillin and kanamycin) were included in addition to methylated compounds (Table 6.3). The antibiotics ampicillin and kanamycin (100 µg/ml) were simultaneously added to investigate the activity of bacteria in the presence of added DMS and DMSP. Antibiotics, either singly or combination, are frequently used to enrich cultures of methanogens from the environment. Due to the cell wall structure associated with all Archaea, methanogens are unaffected by common antibiotics that can be employed as selective inhibitors of bacteria (Godsy, 1980; van der Maarel et al., 1995).

Molybdate is a competitive inhibitor of sulphate reduction and was added at 20 mM final concentration. It is a commonly used inhibitor to study sulphate reduction and its early use confirmed that sulphate reducers compete with methanogens for the hydrogen present in the sediments (Oremland and Taylor, 1978). One possible detrimental side effects of molybdate is the interaction with free thiols and sulphide, resulting in higher redox potentials with an adverse effect on methanogens (Lyimo et al., 2002). However, there was no evidence in this study to suggest that any of the slurry microcosms containing molybdate (benthic and pelagic DMS, DMSP samples) were adversely affected.

**Table 6.3** List of treatments and sampling occasions for benthic and pelagic samples.

Pelagic samples		Benthic samples	
Treatment	Date	Treatment	Date
No amendment	May 20, June 8, Aug 11	No amendment	May 18, Aug 20
+ DMS (20 nM)	June 8, Aug 11	+ DMS (20 nM)	Aug 20
+ DMS + Molybdate	Aug 11	+ DMS + Molybdate	Aug 20
+ DMS + Antibiotics	June 8	+ DMSP (20 nM)	May 18, Aug 20
+ DMSP (20 nM)	May 20, June 8, Aug 11	+ DMSP + Molybdate	Aug 20
+ DMSP + Molybdate	May 20, Aug 11	+ GBT (20 nM)	May 18
+ DMSP + Antibiotics	June 8	+ GBT + Molybdate	May 18
+ DMSO (20 nM)	June 8		

**Figure 6.2** Map of Loch Creran, west coast of Scotland, showing the location of pelagic sediment trap (A); benthic samples: May 18th (B) and August 20th (C).

## 6.3 Results

### 6.3.1 Methane production in copepod faecal pellets

Methane was detected in the headspace of all the samples of faecal pellets from *T. longicornis*. No methane was detected in the control samples. Table 6.4 shows the methane concentrations observed after 7 days of incubation under anaerobic conditions. In the unamended samples of faecal pellets, an average concentration of 12.2 nmol CH<sub>4</sub> dm<sup>-3</sup> was observed. The addition of DMSP resulted in a slight increase, with an average concentration of 15.2 nmol CH<sub>4</sub> dm<sup>-3</sup>.

**Table 6.4** Methane concentrations in the faecal pellets of *T. longicornis* incubated anaerobically for 9 days, both unamended and with the addition of DMSP (20 nM). Methane concentrations are reported as nmol CH<sub>4</sub> dm<sup>-3</sup> of sample media and pmol CH<sub>4</sub> faecal pellet<sup>-1</sup>.

Sample	CH <sub>4</sub> concentration nmol CH <sub>4</sub> dm <sup>-3</sup>	Average CH <sub>4</sub> concentration pmol CH <sub>4</sub> faecal pellet <sup>-1</sup>
Unamended	12.2 ± 1.9	20.3 ± 3.2
+ DMSP	15.2 ± 2.0	27.3 ± 3.3

### 6.3.2 Background levels of biogenic sulphur and methane

The concentrations of biogenic sulphur (total DMSP+DMS) in pelagic material collected in the sediment trap were 1.49, 2.56 and 2.98 µg S g wet sediment<sup>-1</sup> for May 20<sup>th</sup>, June 8<sup>th</sup> and August 11<sup>th</sup> respectively (Table 6.5). These concentrations in the benthic samples were 0.58 and 0.22 µg S g wet sediment<sup>-1</sup> for May 18<sup>th</sup> and August 20<sup>th</sup> respectively.



**Table 6.5** Biogenic sulphur (DMSP+DMS) concentrations in sample material collected from the benthic and pelagic environment in Loch Creran on different sampling occasions.

Pelagic habitat		Benthic habitat	
Sampling date	$\mu\text{g S g wet sediment}^{-1}$	Sampling date	$\mu\text{g S g wet sediment}^{-1}$
May 20 <sup>th</sup>	1.49	May 18 <sup>th</sup>	0.58
June 8 <sup>th</sup>	2.56	August 20 <sup>th</sup>	0.22
August 11 <sup>th</sup>	2.98		

The concentrations of methane in seawater samples collected from the sediment trap receivers after deployment in June and August are shown in Table 6.6. Values range from 141 to 201  $\text{nmol CH}_4 \text{ dm}^{-3}$ .

**Table 6.6** Methane concentrations in seawater collected in the sediment trap receivers.

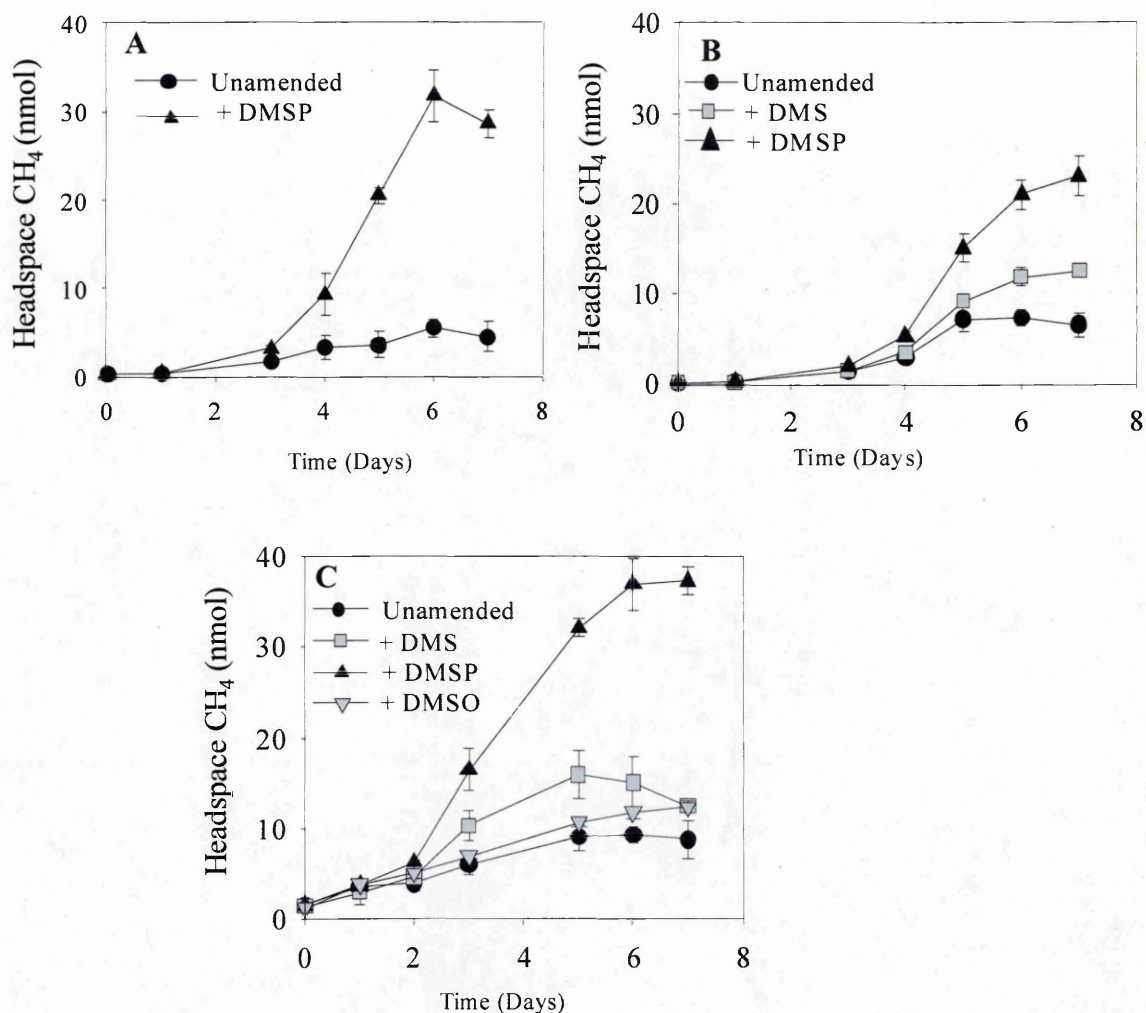
Sampling date	Methane concentrations $\text{nmol CH}_4 \text{ dm}^{-3}$
May 20 <sup>th</sup>	201.8
June 8 <sup>th</sup>	141.1
August 11 <sup>th</sup>	162.9

The sample material collected from the pelagic environment was particularly porous and 1 g of wet weight material collected in the sediment trap was equal to 0.44 g dry weight (56 % water content). In contrast to pelagic samples, benthic sediments were more compact and 1 g of wet weight benthic sediment equalled 0.69 g dry weight (31 % water content).

### 6.3.3 Slurry microcosms

#### *Pelagic material*

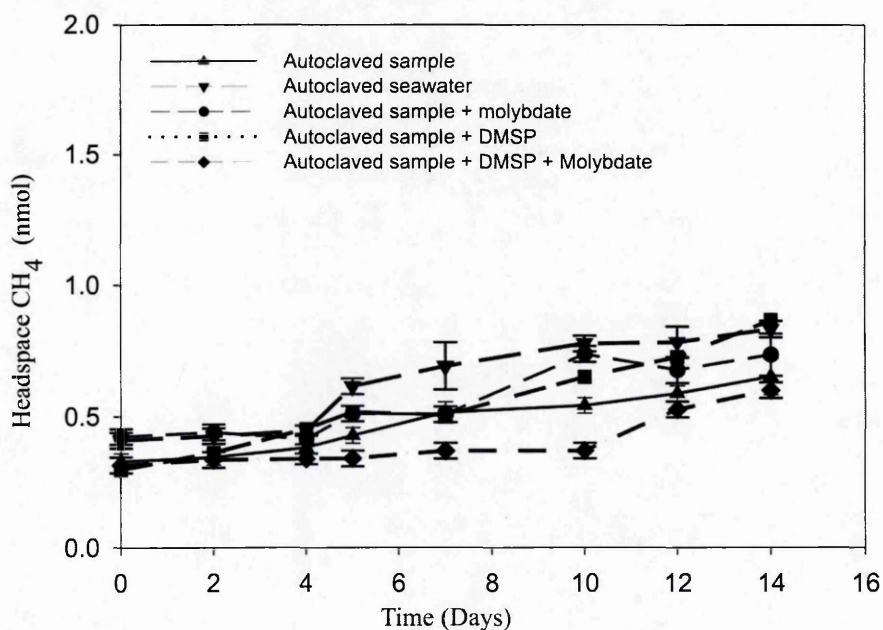
During the anaerobic incubation of particulate material collected from the pelagic environment an increase in the levels of methane was observed after 2 days (Fig. 6.3). In the unamended samples, methane production steadily increased until 5-7 days and then levelled off. The maximum concentration of headspace methane observed was ~9 nM in the unamended samples. Figure 6.3 shows the addition of the methylated sulphur compounds: DMSP, DMS and DMSO to pelagic particulate material. The inclusion of each compound separately stimulated an increase in methane above ambient levels. The addition of DMS in June 8<sup>th</sup> and Aug 11<sup>th</sup> samples produced a ~0.6-fold increase in methane production. The addition of DMSP had a greater effect on methanogenesis with a 3.5 to 5-fold increase in headspace methane observed compared to un-amended samples. This represented a 1.8 to 2.8-fold increase compared to DMS-amended samples on June 8<sup>th</sup> and Aug 11<sup>th</sup> (Fig. 6.3). DMSO also increased methane concentrations when it was added. Although DMSO did not cause such a big increase in methane production as DMS (Fig. 6.3), concentrations continued to rise throughout the experimental period.



**Figure 6.3** Headspace accumulation of methane in slurries of pelagic sample material collected on the a) May 20th; b) June 8th; and c) August 11<sup>th</sup>. Samples were unamended (●); amended with DMS (◻); DMSO (◻); and DMSP (▲). The results are the means for triplicate slurries for each treatment, with standard error bars. Where error bars are not visible, they are smaller than the symbols.

No significant production of methane was observed in the control samples (Fig 6.4).

Figure 6.4 shows the control samples conducted for the pelagic material on May 20<sup>th</sup>. It should be noted that the y-axis is on a different scale to the other results shown for May 20<sup>th</sup> (shown in Fig. 6.3 and Fig. 6.6) to allow the different controls to be discriminated. These control samples are typical for all other pelagic and benthic sampling occasions, and therefore the controls from the other occasions are not shown.

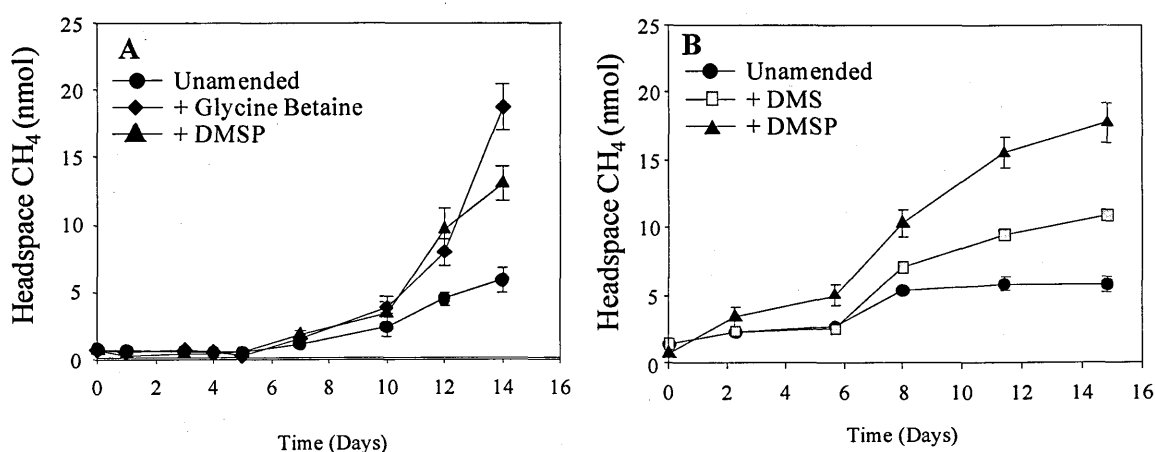


**Figure 6.4** Control samples for May 20<sup>th</sup> from the pelagic environment. Note the y-axis is on a different scale to other results shown from this sampling period.

*Benthic sediments*

An increase in levels of methane was observed after 6-7 days of incubation (Fig. 6.5).

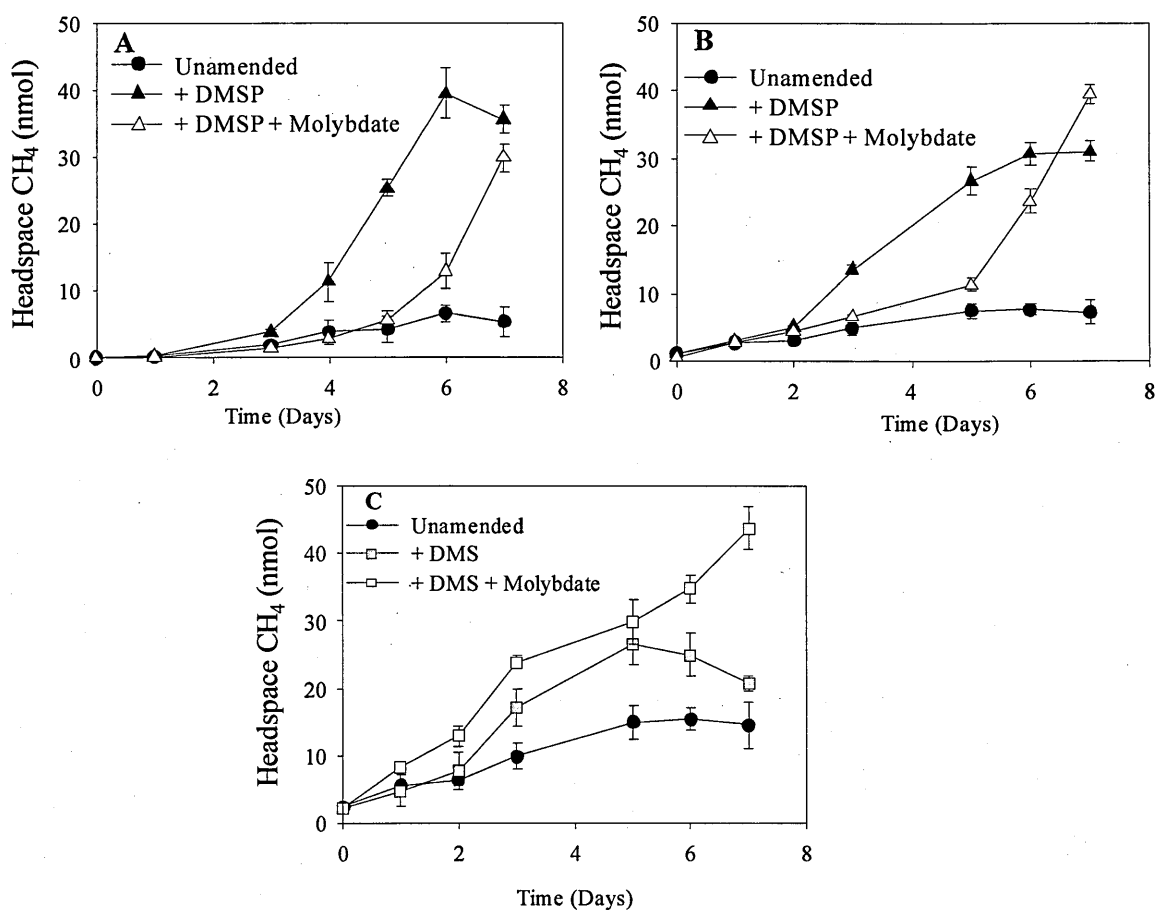
Compared to the pelagic samples, methane concentrations were lower in the unamended samples. The addition of the methylated compounds: GBT; DMSP; and DMS, resulted in an increase of methane production (Fig. 6.5). On May 18<sup>th</sup>, the inclusion of DMSP and GBT caused a 2-3 fold increase in headspace methane, with a greater increase in the GBT-amended sample at the end of the incubation period. On August 20<sup>th</sup>, DMSP and DMS caused a 3-fold and 2-fold increase in respective headspace methane concentrations



**Figure 6.5** Headspace accumulation of methane in slurries of benthic sample material collected on the a) May 18<sup>th</sup> and b) August 20<sup>th</sup>. Samples were unamended (●); amended with DMS (◻); Glycine Betaine (◆); and DMSP (▲). The results are the means for triplicate slurries for each treatment, with standard error bars. Where error bars are not visible, they are smaller than the symbols.

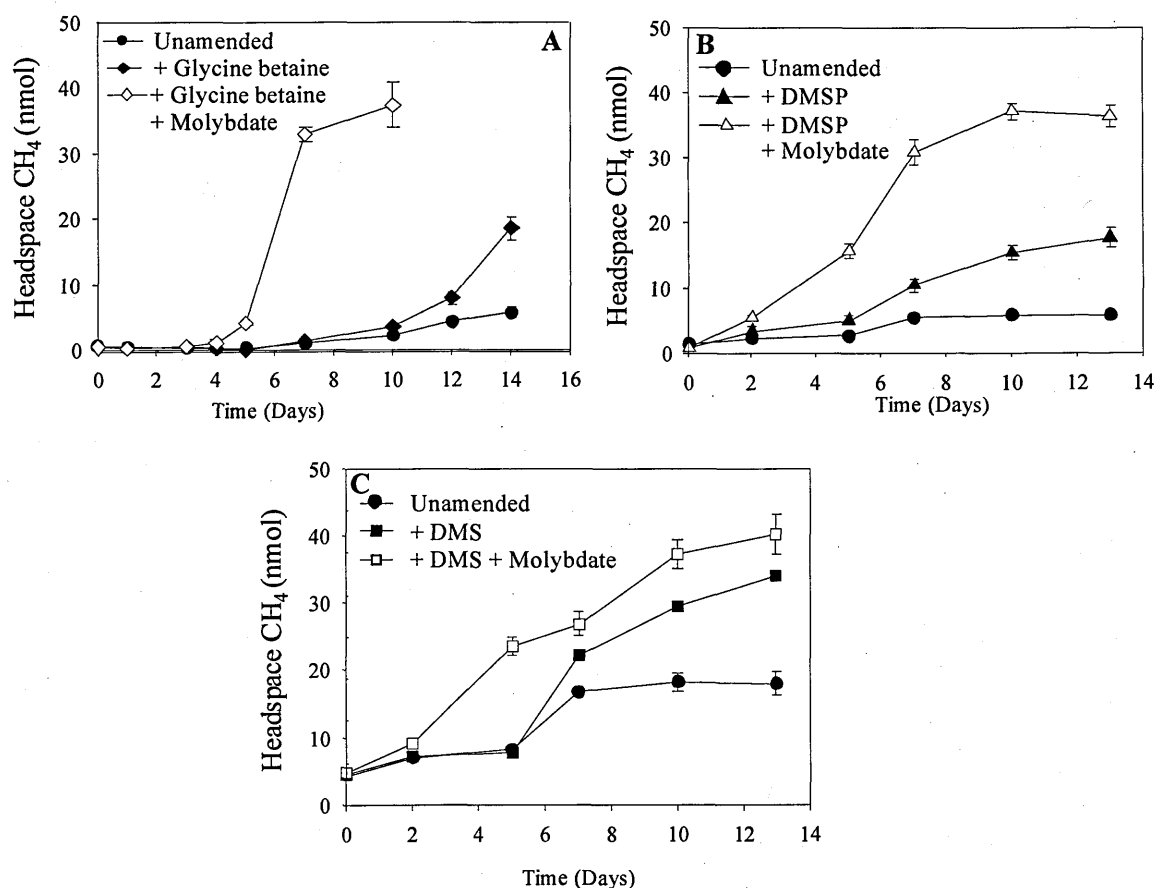
### Amendments - Molybdate

Molybdate was included on two occasions (May 20<sup>th</sup> & August 11<sup>th</sup>) to pelagic material to investigate the role of sulphate reducing bacteria. The addition of molybdate to DMS-amended samples (Fig. 6.6c) resulted in greater levels of methane than just DMS-amended samples (Fig. 6.6c) resulted in greater levels of methane than just DMS-amended alone. This was evident from Day 2 of the incubation period. In contrast, the addition of molybdate to DMSP-amended samples (Figure 6.6a,b) resulted in a 3-4 day increase in lag time before an increase in methane concentrations was observed.



**Figure 6.6** Headspace accumulation of methane in pelagic sample slurries with the addition of molybdate: a) May 20<sup>th</sup>; b) August 11<sup>th</sup>; c) August 11<sup>th</sup>, DMS+molybdate. Samples were unamended (●); amended with DMSP (▲); DMSP+molybdate (△); DMS (◻); and DMS+molybdate (◻). The results are the means for triplicate slurries for each treatment, with standard error bars. Where error bars are not visible, they are smaller than the symbols.

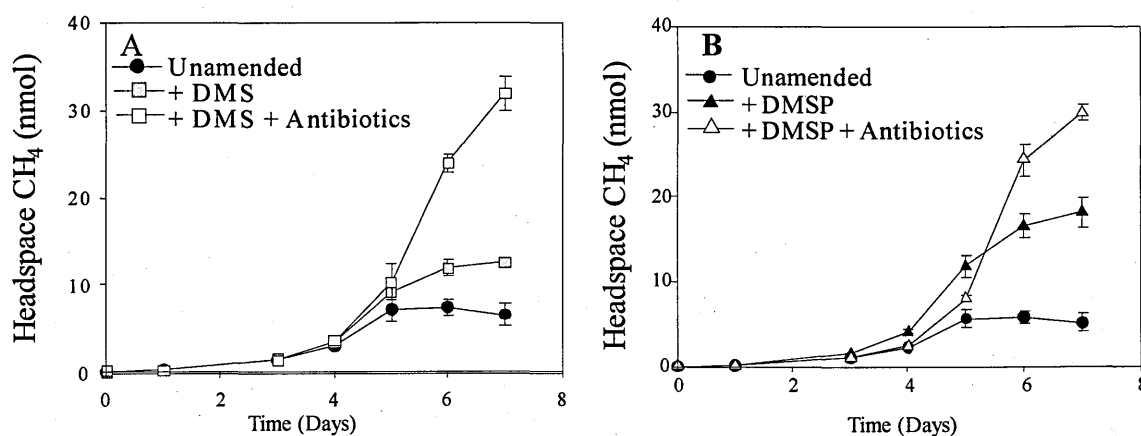
Molybdate was also added to the benthic samples on both sampling dates (May 18<sup>th</sup> & August 20<sup>th</sup>). The addition of molybdate to DMS-amended benthic samples produced a similar result to the pelagic samples with an increase in methane compared to the DMS-only sample (Fig. 6.7c). The addition of molybdate to DMSP- and GBT-amended samples resulted in an increase in methane concentrations greater than the substrate only amendments (Fig. 6.7a,b). A significant decrease was also observed in the lag time before methane concentrations began to rise, which is in direct contrast to the effect of molybdate on DMSP-amended pelagic samples.



**Figure 6.7** Headspace accumulation of methane in benthic sample slurries with the addition of molybdate: a) May 18<sup>th</sup>; b) August 20<sup>th</sup>; c) August 11<sup>th</sup>. Samples were unamended (●); amended with Glycine betaine; Glycine betaine+molybdate (◊); DMSP (▲); DMSP+molybdate (Δ); DMS (■); and DMS+molybdate (□)

### Amendments - Antibiotics

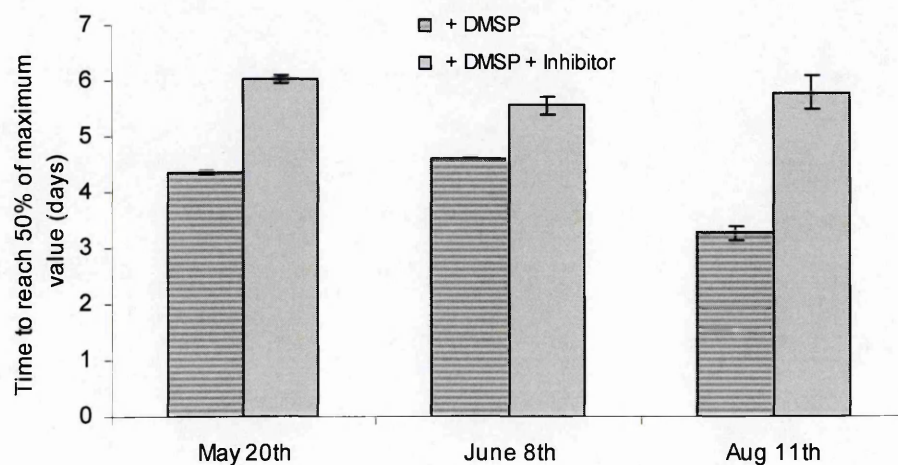
Antibiotics were added to both DMS- and DMSP-amended pelagic samples on June 8<sup>th</sup>. The addition of antibiotics to DMS-amended samples had a positive effect on methanogenesis with an increase in methane concentrations observed towards the end of the incubation period (Fig. 6.8a). The addition of antibiotics to DMSP-amended samples increased the time before an increase in methane concentrations was observed by 1-2 days (Fig. 6.8b). The significance of the delay in methane concentrations compared to the control sample is discussed in context with the other inhibitor manipulations (Fig. 6.9)



**Figure 6.8** Headspace accumulation of methane in pelagic sample slurries with the addition of antibiotics: a) June 8th, DMS + antibiotics; b) June 8th, DMSP + antibiotics. The results are the means for triplicate slurries for each treatment, with standard error bars. Where error bars are not visible, they are smaller than the symbols.



To investigate the statistical significance of including either molybdate or antibiotics in pelagic samples amended with DMSP, a two-stage statistical analysis was performed on the data. A polynomial regression line was applied to six individual samples: May 18<sup>th</sup>: DMSP and DMSP+Molybdate; August 11<sup>th</sup>: DMSP and DMSP+Molybdate; June 18<sup>th</sup>: DMSP and DMSP+Antibiotics. The regression line was then used to calculate the time taken to reach 50% of the maximum value achieved during the experimental period which is subsequently plotted as average  $\pm$  standard error for the three experiments (Fig. 6.9). A 2-sample t-test was subsequently performed on the transformed data to reveal that on all three occasions there was a significant difference between the samples that contained DMSP and those which contained DMSP and inhibitors (May 20<sup>th</sup>:  $F_{2,2} = 36.9$ ;  $p < 0.001$ ; Jun 11<sup>th</sup>:  $F_{2,21} = 13.8$ ;  $p = 0.016$ ; August 8<sup>th</sup>:  $F_{2,21} = 7.81$ ;  $p = 0.001$ )



**Figure 6.9** Comparison of the time taken to reach 50% for the pelagic samples containing DMSP and inhibitors: May and August: DMSP + Molybdate; June: DMSP + Antibiotics. The results are the means for triplicate slurries for each treatment, with standard error bars.

## 6.4 Discussion

### 6.4.1 Production of methane in copepod faecal pellets and particulate material from the pelagic environment

The presence of methane in the headspace of anaerobic microcosms containing copepod faecal material confirms the presence of viable methanogens within *T. longicornis* faecal pellets. Whilst the addition of DMSP was not necessary to produce a detectable signal, its presence did produce an overall higher quantity of methane. These results confirm previous observations of methanogenesis associated with anaerobic incubations of copepod faecal material (Bianchi et al., 1992; Marty, 1993; Marty et al., 1997). For example, Marty et al. (1997) inoculated multiple hungate tubes with zooplankton faecal pellets and analysed them weekly for headspace methane over a 2 month period.

Methane was undetectable in over half of the samples, 6 samples had trace quantities of methane, whilst 3 samples had substantial quantities of methane (80 to 125 nmol CH<sub>4</sub> tube<sup>-1</sup>). In contrast to previous findings by Marty et al. (1997), all of the samples in this study produced methane. The difference in results may have been due to the addition of a reducing agent, which was included to achieve the reduced conditions necessary for methanogenesis (Eh less than -300 mV) (Oremland, 1979).

Methane was also produced during the anaerobic incubation of particulate material collected from the pelagic environment. The pelagic material was not characterised as part of this work and therefore it was assumed to be composed of material including faecal pellets and algal detritus. Previous analysis of sediment trap material has also revealed methanogen activity in samples analysed both *in situ* (Karl and Tilbrook, 1994)

and also using anaerobic incubations similar to this study (Bianchi et al., 1992; Marty et al., 1997)..

For both copepod faecal pellets and pelagic samples, it should be stressed that these results do not provide information on methane production *in situ* because of the necessary handling of samples. The samples were incubated under anaerobic conditions for a period of days. However, the measurable production of methane indicates the potential for methanogenesis given the right conditions within the oxygenated water column.

One of the most comprehensive experiments investigating the production of methane associated with copepod grazing was conducted by de Angelis and Lee (1994) using  $^{14}\text{C}$ -labelled phytoplankton. However, even with the detection of  $^{14}\text{C}$ -methane, the exact location of methanogenesis was not resolved and three possible sites were postulated for methane production: algal cells, copepod digestive tracts and faecal pellets (de Angelis and Lee, 1994). This highlights the limitation of working with incubated material to resolve processes occurring within the upper water column and it is suggested that a greater understanding of the methanogen community composition and physiology is required. An analysis of copepod faecal pellets and digestive tracts for methanogens was conducted in Chapters 7 and 8.

#### 6.4.2 Ambient concentrations

##### *DMSP+DMS*

The ambient biogenic sulphur (DMSP+DMS) concentrations in the benthic sediments, ranged from 0.22 to 0.58  $\mu\text{g S g wet sediment}^{-1}$  which equates to 5.1 to 13.5  $\mu\text{mol S dm}^{-3}$

wet sediment (assuming 1 cm<sup>3</sup> sediment from Loch Creran represents ~1.34 g of wet sediment) (Table 6.5). These biogenic sulphur values are at the lower end of DMSP and DMS values previously recorded at the surface of sediments in Loch Creran of 5.3 to 89.3  $\mu\text{mol S dm}^{-3}$  wet sediment (Hughes et al., 2004). In sediment trap material, biogenic sulphur concentrations ranged from 1.49 to 2.98  $\mu\text{g S g wet sediment}^{-1}$  which represents 62.4 to 124.7  $\mu\text{mol S dm}^{-3}$  wet sediment (Table 6.5). The higher concentrations of biogenic sulphur in surface material reflects the upper water column as the site of algal-DMSP production. With such high concentrations of ambient DMSP+DMS, it is somewhat intriguing that the addition of DMSP and DMS at 20 nmol dm<sup>-3</sup> (final concentration) in slurry microcosms stimulated methane production above ambient levels. It suggests that the biogenic sulphur contained in the material is not metabolically available to methanogens, either because it is utilised by other microbes or because it is intracellular (bacteria, protists or algae). To investigate this further a replicate treatment of the slurry microcosms should contain material that has been sonicated to physically disrupt the cells, thereby releasing DMSP to the wider media.

### *Methane*

Methane concentrations were measured in seawater retained in the sediment trap (Table 6.6). The concentrations recorded in this study exceed the methane concentrations (1.1-56.6 nmol dm<sup>-3</sup>) measured in sediment traps which were deployed in the oligotrophic North Pacific by Karl and Tilbrook (1994), even though their traps were deployed for up to 22 days. The higher concentrations of methane recorded in this study are a likely consequence of increased productivity and organic material associated with the coastal environment (as discussed in Chapter 3). The production of methane in sediment traps

provides additional support to methanogenesis occurring in sinking particulate material and provides an explanation for the elevation in methane concentrations at the pycnocline (e.g. Scranton and Brewer, 1977). It should be noted that the measurements of methane in sediment trap seawater are not comparable to ambient levels of methane in the water column. In general, the coastal environment and nearshore waters are associated with high concentrations of methane due to *in situ* production and also due to the transport of methane from riverine input (Upstill-Goddard et al., 2000; Middelburg et al., 2002).

### 6.4.3 Slurry microcosms

#### *Pelagic samples*

The addition of DMSP and DMS to pelagic particulate material stimulated methane production compared to the unamended samples on all sampling occasions. This finding highlights the role of methylated sulphur compounds as potential precursors to methanogenesis in the pelagic environment. This has previously been reported for other anaerobic environments, such as salt marsh sediments (Kiene and Visscher, 1987); marine sediments (Kiene and Taylor, 1988) and freshwater sediments (Lomans et al., 1999). It has previously been observed in Chapter 3 that levels of DMSP and DMSO associated with algae cells can extend up into the millimolar range on a per litre of cell volume basis. In addition, analysis of DMSP in copepod faecal pellets demonstrated concentrations of the micromolar level on a per litre of faecal pellet scale (Chapter 4). These hotspots of DMSP concentrations in the pelagic environment suggest that any localised depletion of oxygen in particulate material such as faecal pellets of algal debris, could facilitate the metabolism of DMSP by methanogens.

The availability of DMSP and DMS as metabolic substrates for methanogens is comparable to the nitrogen analogues glycine betaine and methylamines (King, 1984a). Neither DMSP, nor glycine betaine can be metabolised directly by methanogens, but the derivative compounds *e.g.* DMS and methylamines can be utilised. This was exemplified in a co-culture of the methanogen *Methanosarcina barkeri* and the anaerobic bacteria *Clostridium sporogenes*, when betaine was fermented by *C. sporogenes* and *M. barkeri* metabolised the trimethylamine which was subsequently produced (Naumann et al., 1983). These results highlight the importance of the wider anaerobic microbial community in the degradation of compounds, which is discussed in further detail with regards to the inhibitor studies.

In addition to DMSP and DMS, the inclusion of DMSO also increased the production of methane, although not to the same extent (Fig. 6.3c). Select bacteria and haloarchaea can use DMSO as an electron acceptor during anaerobic respiration (McCrindle et al., 2005; Muller and DasSarma, 2005). However, at present there is no evidence that DMSO reduction extends to methanogenic archaea and it is probable that methane production was stimulated by the formation of DMS resulting from DMSO reduction by other anaerobes, as previously suggested by Kiene and Capone (1988). One example of this was highlighted by the syntrophic relationship between *Syntrophomonas erecta* and *Methanobacterium formicum*. *S. erecta* used DMSO as an electron acceptor during growth on the fatty acid butyrate in the presence of *M. formicum* (Wu et al. 2006). Short chain fatty acids, *e.g.* butyrate, are degraded by at least two trophic groups because of the unfavourable energetics of the reactions (Schink, 1997) and the H<sub>2</sub> scavenger was the methanogen *Methanobacterium formicum* (Wu et al., 2006).

*Benthic samples*

In slurry microcosms containing sample material from benthic sediments, lower rates of methane production were observed in unamended samples compared to the unamended pelagic samples. This may reflect the more refractory nature of organic matter associated with these habitats. The addition of DMS, DMSP and GBT increased the levels of methane, as previously observed in sediment environments (King, 1984a; Kiene and Visscher, 1987; Kiene and Taylor, 1988). A comparison of the benthic and pelagic slurry microcosms reveals a longer lag time is observed in the benthic samples before an increase in methane concentrations is observed. The reason for the longer lag time is unclear at present. It may reflect a lower activity of methylotrophic methanogenesis in the sediment or a greater competition between sulphate-reducers and methanogens in the benthic environment. The delay could also be due to exposure of the methanogenic community to oxygen during the establishment of the microcosms. These results highlight the lack of knowledge that exists with regards to the response of methanogens to changing redox conditions. Whilst the potential for particulate material to become anaerobic has been thoroughly reviewed (Alldredge and Silver, 1988; Shanks and Reeder, 1993; Ploug et al., 1997; Schramm et al., 1999), it has not been widely considered from the view-point of the methanogens. To date, methanogens appear to have retained their status as 'fastidious anaerobes', whilst other anaerobic bacteria such as sulphate-reducing bacteria, have been investigated for their tolerance and mechanisms for dealing with oxygen stress (Cypionka, 2000).

#### 6.4.4 Effect of inhibitors on methane production

##### *Molybdate and DMS*

The addition of molybdate to DMS-amended samples caused a greater increase in methane concentrations in both benthic and pelagic samples, compared to the exclusive addition of DMS. The increase in methane concentrations indicate that methanogens have to compete for DMS with sulphate-reducers. Previous work has shown that the addition of sulphate to slurry experiments of marine sediments caused a decrease in DMS consumption (Kiene and Visscher, 1987). A pure culture of DMS-utilising sulphate-reducer has been isolated from a thermophilic digester (Tanimoto and Bak, 1994).

A comparison of the thermodynamics involved in the metabolism of methylated compounds by sulphate-reducers and methanogens is provided by Scholten et al. (2003). A comparison of the calculated  $\Delta G^{\circ}$  for DMS respired by sulphate-reducers and methanogens is -92.2 and -73.8 respectively, indicates SRB's gain more energy from the metabolism of DMS (Scholten et al., 2003). This is an important consideration as methylated compounds are sometimes referred to as 'non-competitive compounds' due to the concomitant use by methanogens and SRB's. However this appears to only occur at high ( $>\mu\text{mol}$ ) concentrations of DMS (Lomans et al., 2002). At lower concentrations, such as levels of DMS typically observed in the upper ocean ( $\sim 5 \text{ nM}$ ) sulphate reducers have increased affinity for DMS (King, 1984b; Kiene et al., 1986) and will compete with methanogens.



*Molybdate and DMSP*

The addition of molybdate to DMSP-amended samples also influenced the production of methane though there was a different response between sample material collected from the benthic and the pelagic environment. The addition of molybdate to pelagic material caused a delay in methane production relative to the uninhibited sample, whilst in benthic sediment samples, molybdate stimulated an increase in methane concentration. The latter observation can be explained by the removal of competition between sulphate-reducers and another microbe. In anaerobic environments methanogenesis is usually higher when sulphate concentrations are low or if sulphate reducers have been artificially inhibited (Banat et al., 1983). The former observation can be explained by a requirement by the methanogens on sulphate-reducers. Sulphate-reducers can degrade DMSP and the derivative compounds (*e.g.* 3-methiolpropionate, methanethiol, DMS) can be subsequently metabolised by methanogens (Fig. 6.10). This has previously been demonstrated for other methylated compounds including methionine (Kiene and Visscher, 1987) and glycine betaine (King, 1988). To confirm this possibility, a further control consisting of an unamended sample with the addition of molybdate is required. This would further reveal the effect of sulphate-reducers on the addition of DMSP.

The possibility that methanogens depend on sulphate-reducers contrasts with previous suggestions that methane production in the sulphate-rich surface ocean is methylotrophic to avoid competition with sulphate-reducing bacteria. An important point to highlight is the lack of knowledge on sulphate-reduction within anaerobic micro-niches in the upper water column. Within the pelagic environment, sulphate reduction has only been reported for the anoxic bottom waters of Big Soda Lake (Smith and Oremland, 1987).

The production of sulphide has also been demonstrated in marine particulate material (Shanks and Reeder, 1993), however this has not been universally observed (Schramm et al., 1999). However it should be made clear that overall our understanding on sulphate-reduction in micro-aerobic environments is increasing. Over the last two decades, researchers have revealed the ability of sulphate-reducers to survive in oxic conditions and even respire oxygen and nitrate (Cypionka et al., 1985; Krekeler and Cypionka, 1995). Sulphate-reducers have also been found to have a wide array of adaptive mechanisms for tolerating free radicals produced under oxic conditions (Fournier et al., 2003).

### *Antibiotics*

The addition of antibiotics to the pelagic material slurry microcosms produced a similar result to the inclusion of molybdate. The inclusion of antibiotics with DMS caused a greater increase in methane than just DMS alone indicating methanogens compete with bacteria for DMS. DMS has been identified as a metabolic substrate for bacteria and anoxygenic phototrophs (Visscher and van Gemerden, 1991; Lomans et al., 1999) and sulphate-reducing bacteria (Tanimoto and Bak, 1994). In contrast, the addition to DMSP-amended samples significantly increased the lag time before methane concentrations increased. This suggests that members of the Archaea can degrade DMSP in addition to the bacteria listed above. If the Archaeal degradation of DMSP did not occur, then an absence of methane production would have occurred. Although DMSP-degradation may have been carried out by an Archaeal sulphate-reducer, this is unlikely as to date there is only one report of a sulphate-reducing Archaea and it is a thermophile which normally functions at temperatures between 60-90 °C (Castro et al., 2000). It is

possible that the Archaea responsible was a Euryarchaea or Crenyarchaea that have recently been discovered in the water column (DeLong, 2003).

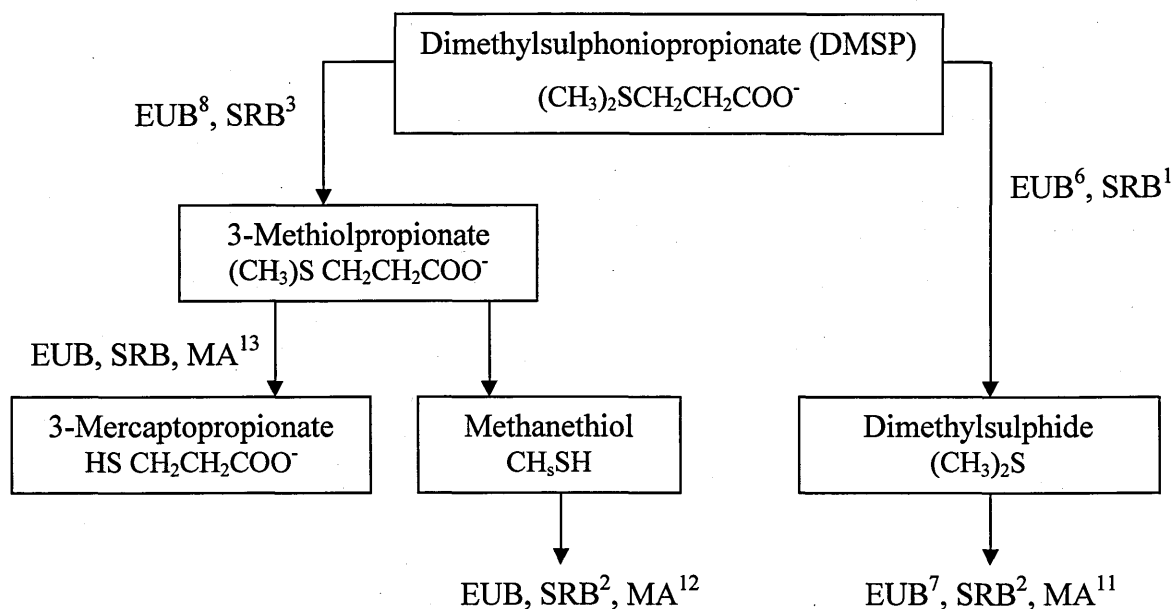
It should be noted that the extrapolation of data from slurry experiments to water column process should be conducted very carefully due to the absence of redox gradients and other parameters that influence *in situ* bacterial activity. The inhibitor experiments provide information on the presence and potential role of the sulphate-reducers and other bacteria. The activity of different functional groups can be explored further through appropriate molecular techniques. For example, the spatial assembly of community could be studied by *in situ* hybridisation techniques (Pernthaler and Pernthaler, 2005).

#### 6.4.5 Conversion of DMSP to methane

Although this study did not elucidate the pathway by which DMSP is converted to methane, there is sufficient evidence in the literature to speculate on the various mechanisms. Figure 6.10 highlights the possible routes for DMSP degradation and key derivative compounds: DMS; methanethiol and 3-methylpropionate, though the interactions between these derivatives are not highlighted. Also highlighted are the examples of the micro-organisms identified for the different pathways.

It is evident from Figure 6.10 that the derivatives of DMSP are methanogenic precursors, but not DMSP itself. Therefore, DMSP can be cleaved to DMS and acrylate by both SRBs and bacteria but not methanogens. Subsequently, DMS is used either by methanogenic archaea (as listed in Table 6.2), SRBs or bacteria. Alternatively DMSP is

demethylated to 3-methylpropionate by SRBs and bacteria, however once more, not by methanogenic archaea. 3-methylpropionate is typically portrayed as an intermediary compound which can be degraded further to 3-mercaptopropionate and methanethiol. However whilst methanogenic archaea can convert 3-methylpropionate to 3-mercaptopropionate, no anaerobic micro-organisms has been isolated that can completely demethylate DMSP (Scholten et al., 2003). This work affirms that methanogens predominantly exist as a component of wider microbial consortia. The presence of a wider anaerobic food web provides additional support for methanogenesis in particulate material in the surface waters of the ocean. The potential for obligate and facultative syntrophic relationships and competition between microbes involved in upper water column methanogenesis is to date largely uncharacterised, although it has been speculated upon by Sieburth (1991).



**Figure 6.10** Schematic diagram highlighting DMSP degradation and examples of bacteria (EUB), sulphate-reducers (SRB) and methanogens (MA) that metabolise the different compounds. 1: *Desulfovibrio acrylicus* (van der Maarel et al., 1996); 2: *Desulfotomaculum* sp. (Tanimoto and Bak, 1994); 3: *Desulfobacterium* sp. (van der Maarel et al., 1993); 6: *Clostridium propionicum* (Wagner and Stadtman, 1962); 7: *Thiobacillus* sp. (Visscher and Taylor, 1993); 8: *Eubacterium limosum* (Jansen thesis); 11: See Table 2; 12: Strain MTP4 (Finster et al., 1992); 13: *Methanosarcina* sp. (van der Maarel and Hansen, 1997).

## 6.5 Conclusions

This work has demonstrated that methanogens located within particulate material in the pelagic environment can benefit (either directly or indirectly) from the availability of DMSP, DMSO and DMS. In combination with the results from Chapter 3 & 4, this suggests that particulate material in the pelagic environment can play an important role in both the production of methane, a potent greenhouse gas, and also methylated sulphur compounds, which are important precursors to sulphate aerosols. Looking beyond the immediate methanogenesis process, the addition of inhibitors indicates that methanogenic Archaea in the upper water column are part of a wider microbial consortium. For example, sulphate-reducing bacteria can degrade the algal compound DMSP providing metabolic substrates for methanogens. The sulphate-reducing bacteria would also further reduce the conditions for the more fastidious anaerobic methanogens. The details of anaerobic microbial consortia have so far predominantly escaped the attention of scientists exploring the oceanic methane paradox. The recovery of viable methanogens from both copepod faecal pellets and pelagic particulate material indicates that methane can be produced under the necessary conditions in the upper water column. However neither the incubation of sample material, nor the presence of methanogens, reveals the mechanism by which conditions are produced for *in situ* methanogenesis. To better understand this process, an initial study of the methanogen community diversity using 16S rRNA gene analysis was undertaken (Chapter 7).



## Chapter 7. Methanogenic Archaea in the upper water column

### 7.1 Introduction

Methanogens are an ecologically important group of obligate anaerobes that produce methane as a product of their metabolism through a diverse range of biomes such as wetlands, sediments, termites, and deep-sea hydrates. Methane is also produced in the open oceans and whilst the amount is a comparatively small source of the total global methane produced (10-15 Tg/yr) (Fung et al., 1991), its production in the oceans remains enigmatic and not well-understood. Methane concentrations in the upper water column are frequently supersaturated (2-4 nM) with respect to the atmospheric equilibrium (Scranton and Brewer, 1977; Ward et al., 1987; Owens et al., 1991; Oudot et al., 2002). The supersaturation of methane is considered to result from biological methanogenesis, however the question of how obligate anaerobic methanogens can function in the oxygenated water column is considered a biochemical and microbial paradox (Kiene, 1991). One of the reasons why methanogenesis in the pelagic environment remains unresolved is the great difficulty in correlating measured methane concentrations with *in situ* methanogen activity and the clear identification of methanogen diversity.

Methanogens are unable to directly utilise complex organic molecules for energy generation, but instead rely on fermentative and respiratory organisms to provide them with low molecular weight compounds (Zinder, 1993). The metabolic pathways of cultivable methanogens are well described and traditionally have been used to taxonomically delineate methanogens into three functional groups based on substrate use: the reduction of methylated compounds; CO<sub>2</sub> reduction; and acetate disproportionation. In the oceans, it is considered likely that methane production occurs via the metabolism of



methylated compounds, which in the context of the upper ocean, is likely to be derived from phytoplankton fixed carbon, and include compounds such as methylamines, methanethiol and dimethylsulphide (Cynar and Yayanos, 1991; Sieburth and Donaghay, 1993). In support of this hypothesis, the three earlier reports describing methanogenic Archaea from the upper ocean have all been related to the Methanosarcinaceae family, which are known for their ability to solely metabolise methyl compounds. Cynar and Yayanos (1991) isolated *Methanococcoides methylutens* from the marine plankton in the East Pacific. Sieburth (1993) isolated four clusters of methylotrophic methanogens from samples obtained from the oxygenated water column of Chesapeake Bay. The clusters were related to *Methanosarcina barkeri*, *Methanosarcina mazei*, *Methanosarcia* sp. and the fourth clade was restricted to deeper waters and unrelated to extant methanogens. More recently, van der Maarel et al. (1999) identified the presence of *Methanococcoides methylutens* within small particles on the North Sea water column using 16S rDNA methodology.

In addition to the isolation and identification of methanogens, methane production has also been measured on other occasions in similar samples collected from the pelagic environment. Oremland (1979) observed the methane production during the anaerobic incubation of copepod samples. More recently, de Angelis and Lee (1994) observed methane production during the grazing of copepods of phytoplankton in laboratory experiments. Methane was also measured in sinking material collected in sediment traps deployed in the North Pacific (Karl and Tilbrook, 1994). The deployment of sediment traps in the North Atlantic also provided sample material that produced methane when incubated anaerobically (Marty et al., 1997). Specific analysis of copepod faecal pellets

has also revealed methanogenesis (Bianchi et al., 1992; Marty, 1993; Marty et al., 1997). Faecal material is considered to provide an organic-rich habitat and could facilitate the oxygen depletion that is an essential precursor for methanogenesis. On all occasions, the faecal pellets were collected and added to substrate-rich media. Despite these widespread observations of methanogenesis in samples from the pelagic environment, the identification of methanogens in these micro-habitats is under-represented. Furthermore, analysis of faecal pellets from the copepod *Pseudocalanus* sp. did not detect any Archaea using 16S rDNA probes (van der Maarel et al., 1998).

It should also be noted that in the pelagic environment, oxygen depletion within particles is tightly constrained by physical, chemical and biological features of the material such as size, organic matter content and bacterial respiration, and is not generally considered to be sufficient to lead to an appropriate reduction in the redox potential for methanogenesis to occur routinely (Alldredge and Cohen, 1987; Ploug et al., 1997). Consequently, the gut environment of marine copepods and higher trophic organisms has been proposed as the most likely site of methanogenesis in the upper ocean, and would also be a likely inoculum via the release of faecal pellets. Here, the high rates of oxygen consumption during passage of prey items through the gut might produce the reduced conditions favourable for methanogenesis. This process is supported by observations of methane production during incubations of copepods (de Angelis and Lee, 1994), and also in zooplankton faecal pellets (Marty, 1993; Marty et al., 1997). However, the relationship between faecal pellets, gut environment and anaerobes is far from clear, and also whether faecal pellets are an accurate derivative of the gut environment, remains unknown.

This study sought to link previous measurements of methane production in faecal pellet incubations (Bianchi et al., 1992; Marty, 1993; Marty et al., 1997) with the identification of methanogens in particulate material. To investigate if methanogens were present in copepod faecal pellets, we used semi-nested PCR amplification of archeal 16S rDNA. Faecal pellets from two monocultures of copepods were screened and compared with both faecal samples collected from natural assemblages of zooplankton and general particulate material collected from the upper water column. Subsequent to the work conducted with samples collected from Dunstaffnage Marine Laboratory, several European marine institutes were contacted to see if they maintained *Acartia* sp. in culture and could collect faecal material for analysis. The main aim was to corroborate the results obtained from cultures of copepods at Dunstaffnage Marine Laboratory by analysing samples from other marine laboratories

## 7.2 Method

### 7.2.1 Collection of Samples

The samples analysed in this study consisted of copepod faecal pellets, collected from both cultured copepods and 'natural' communities, and uncharacterised particulate material from the upper water column (Table 7.1).

**Table 7.1** Origin of samples and the species of copepod that faecal pellets were collected from.

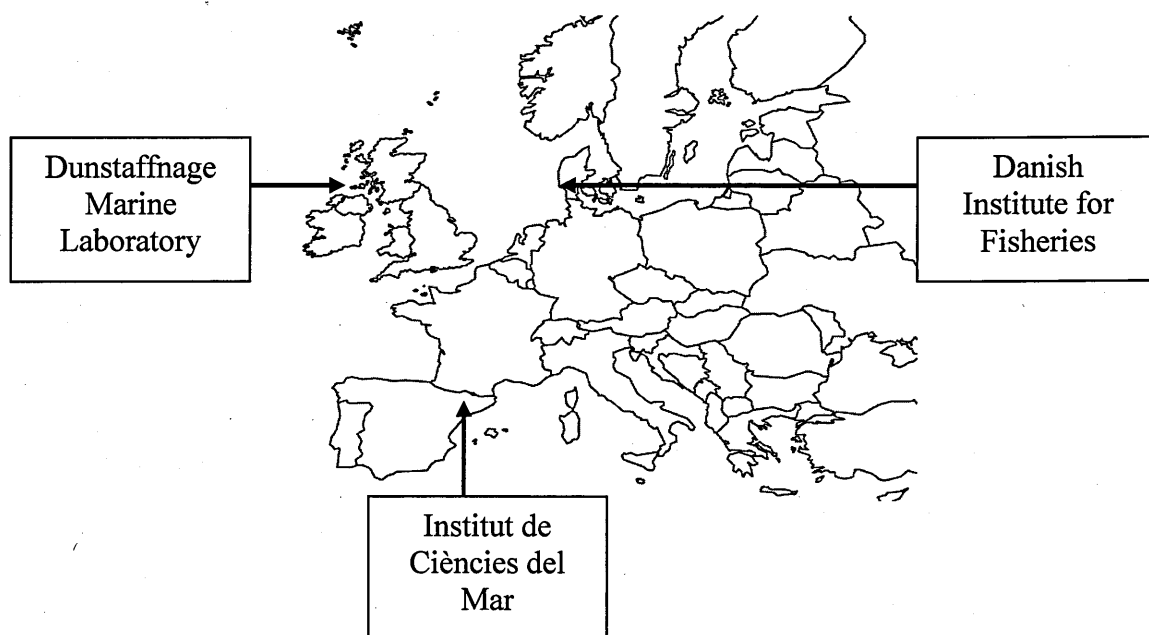
Species	Location of isolation	Prey	Institute
<i>Temora longicornis</i>	Oban, West Scotland	<i>Rhinomonas lacustris</i> , <i>Oxyrrhis marina</i>	Dunstaffnage Marine Lab
<i>Acartia clausi</i>	Oban, West Scotland	<i>Rhinomonas lacustris</i>	Dunstaffnage Marine Lab
<i>Acartia tonsa</i>	Denmark	<i>Rhinomonas salina</i>	Danish Institute for Fisheries
<i>Acartia grani</i>	Barcelona, NW Mediterranean	<i>Rhinomonas salina</i>	Institut de Ciències del Mar
<i>In situ</i> zooplankton community	Loch Linnhe, West Scotland	un-identified	Dunstaffnage Marine Lab
Pelagic particulate material	Loch Creran, West Scotland	-	Dunstaffnage Marine Lab

The adult copepods *Temora longicornis* and *Acartia clausi* used in this study were isolated from a vertical zooplankton trawl in Loch Linnhe, Scotland, using a 68 µm mesh-sized net. The copepods were kept at 14 °C and fed *Rhinomonas* sp. and the heterotrophic dinoflagellate *Oxyrrhis marina*. Both copepod species were maintained in culture for over 6 months and had been through successive generations prior to collection of faecal pellets.

To collect faecal pellet samples from cultured copepods, approximately 300 fresh faecal pellets were collected on a 32 µm mesh, transferred to a glass Petri dish and examined under a light microscope. The faecal pellets were collected by centrifugation at 13,000 x g for 10 min, and stored at -80 °C until used. Field samples were collected from mixed *in*

*situ* zooplankton communities using the 68 µm zooplankton net and placing the organisms in a 10 litre container, maintained at ambient seawater temperature. Faecal pellets were recovered following the procedure outlined for copepod cultures. Samples for sinking particulate material were collected using a sediment trap (see Chapter 5) deployed in Loch Creran and suspended in a water column at a depth of 20 m for 24 hr. The trap material was mixed for 10 min at 150 rpm on an orbital shaker, then washed with sodium phosphate buffer (120 mM, pH 8.0) to remove extracellular nucleic acids (Tsai and Olson, 1991). Samples were then centrifuged at 6,000 x g for 10 min and the washing step repeated twice, before storage at -80 °C.

Faecal pellet samples from *A. clausi* and *T. longicornis* were collected within three hours of defecation, and typically ~0.08 mg (wet weight) of faecal material was used in the DNA extraction process. A similar quantity of faecal material was collected for the field sample, whilst ~10 mg (wet weight) was collected in the sediment trap. For externally sourced samples, faecal material was obtained from cultures of *Acartia* sp. independently collected and maintained in culture at two European research institutes (Fig. 7.1). Faecal pellets were collected as previously described, transported to Dunstaffnage Marine Laboratory in eppendorf's contained with an ice-filled thermos flask and stored at -80°C until analysis. On both occasions duplicate material was collected and analysed.



**Figure 7.1** Map of Europe showing the location of samples for analysis.

### 7.2.2 DNA extraction

An overview of the DNA extraction procedure is provided in Section 2.3. DNA was extracted following the procedure of van der Maarel (1998) (Table 2.1) with a few modifications. During extraction of nucleic acids with phenol-chloroform-isoamyl alcohol (25:24:1), the addition of phenol-chloroform was repeated. The nucleic acids were precipitated with 99 % ethanol and 4.5  $\mu$ l 3 M sodium acetate, gently mixed and incubated on ice for 30 min. The precipitated nucleic acids were centrifuged at 13,000 x g for 20 min and washed in ice-cold 70% ethanol. The samples were then re-pelleted at 13,000 x g for 20 min, dried and re-suspended in 100  $\mu$ l TE (10 mM Tris, 0.1 mM EDTA, pH 8.0) buffer before storage at -80°C.

### 7.2.3 PCR amplification

A two-stage hemi-nested procedure with touchdown amplification, as described in Munson (1997), was performed on five replicates of 1 µl sample to reduce the influence of PCR bias (Wagner et al., 1994). In brief, the PCR mixture (50 µl) contained 5 µl buffer, 4 µl MgCl<sub>2</sub> (25 mM), 2 µl of forward and reverse primer (10 pmol), 2 µl bovine serine albumin (100 µg/ml), 0.5 µl dNTP (20 mM), 1 U *Taq* polymerase (AB gene, UK) and 34.3 µl H<sub>2</sub>O. Primary PCR amplifications used the Archaeal primers 1Af (TCYGKTTGATCCYGSCRGAG) and 1404R (CGGTGTGTGCAAGGRGC) (Embley et al., 1992). PCR amplification involved denaturing at 95 °C for 5 min, followed by 9 primary cycles (94 °C for 1 min, 63 °C for 1 min (decreasing 1 °C per cycle), 72 °C for 2 min and then 24 secondary cycles (92 °C for 30 s, 53 °C for 30 s and 72 °C for 30 s) with a final extension of 72 °C for 5 min followed by cooling at 10 °C for 1 min (PTC 200). The PCR products from the primary amplification were pooled and concentrated to 20 µl with MicroCon 30 cartridges (Millipore, UK). Replicate (five times) 2 µl aliquots were used in the secondary amplifications with the Archaeal primers 1Af and 1100R (TGGGTCTCGCTCGTTG). PCR amplification involved denaturing at 95 °C for 5 min, followed by 9 primary cycles (94 °C for 1 min, 50 °C for 30 s, 72 °C for 2 min) and then 24 secondary cycles (92 °C for 30 s, 55 °C for 30 s and 72 °C for 2.5 min) with a final extension of 72 °C for 5 min followed by cooling at 10 °C for 1 min. *Methanobrevibacter* sp. plasmid (Purdy, K.J.) was used as a positive control and no DNA was added in the negative control. Two negative controls were used in the secondary amplification: a 1 µl aliquot from the negative control used in the primary amplification and a non-DNA control. PCR products were viewed on 0.6% agarose gel by electrophoresis and Ethidium Bromide staining.

### 7.2.4 Cloning procedure

PCR products were ligated into a p-GEM-T vector (Promega, UK) following manufacturers instructions and incubated overnight at 4-5 °C. Ligation products were transformed into *E. coli* DH5α cells on agar medium containing Ampicillin (25 µg/ml), IPTG (160 µM) (Isopropyl β-D-1-thiogalactopyranoside), and X-Gal (200 µM) (5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside). To confirm the presence of an insert, and study the diversity of the inserts, the blue/white screening process was used. Ten colonies were randomly selected and amplified using a 25 µl master mix containing 2.5 µl buffer, 1.8 µl MgCl<sub>2</sub> (25 mM), 0.25 µl of forward and reverse vector-based primers ARD-F and ARD-R primers (Green et al., 2004) (10 pmol), 0.25 µl dNTP (20 mM), 0.5 U *Taq* polymerase and 19.45 µl H<sub>2</sub>O.

### 7.2.5 Restriction Fragment Length Polymorphism (RFLP)

Following successful ligation and transformation, forty clones were selected per environmental sample, the inserts re-amplified with the ARD-F and ARD-R primers (Green et al., 2004) (Table 2.2). The re-amplified PCR-products were digested with the restriction enzyme *RsaI* (Promega, UK). The reaction mixture (20 µl) contained 10 µl PCR product, 1 µl *RsaI*; 2 µl REN buffer, 0.2 µl bovine serine albumin (100 µg/ml) and 5.8 µl H<sub>2</sub>O. The digestion programme was run at 37°C for 15 hours with a final step of 65 °C for 15 min, followed by rapid cooling at 4°C. Digested fragments were viewed with gel electrophoresis using 2.5% Metasieve agarose. The resulting sequences were analysed visually against known *Methanogenium* and *Methanlobus* RFLP patterns and clones displaying distinct RFLP patterns were selected for sequencing.



### 7.2.6 DNA Sequencing

The clones chosen for sequencing were grown in Luria Broth media and the plasmid extracted following the NucleoSpin Plasmid protocol (Abgene, UK). Sequencing mixture consisted of 10 µl BigDye reactions (25% concentration) containing 1 µl M13 primer (forward or reverse), 1 µl H<sub>2</sub>O, 5 µl plasmid, 2 µl BigDye Reaction mix and 1 µl 5x BigDye buffer. The sequence reaction products were incubated for 1 hour at room temperature with 80 µl 75% iso-propyl alcohol (IPA) and 10 µl H<sub>2</sub>O. The precipitated products were pelleted at 13,000 x g for 20 min. The pellet was resuspended in 250 µl IPA and centrifuged at 13,000 x g for 5 mins. The supernatant was carefully discarded and samples dried before storage at 4 °C. Plasmids were sequenced on an ABI-377 DNA sequencer (KBiosciences).

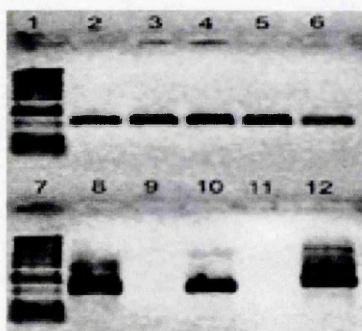
### 7.2.7 Phylogenetic Analysis

The phylogenies were examined by analysis of partial 16S rRNA gene sequences (1080 bases). Sequences of each clone were compared using a BLAST-n analysis on the National Center for Biotechnology Information database (Altschul et al., 1997) to identify most closely related database sequences. Two chimeric clones were detected with RDP software (<http://rdp.cme.msu.edu>) and discarded from subsequent analysis. Sequence alignment (of 1080 basepairs) was performed using MegAlign.

A 16S rRNA gene sequence-based phylogenetic tree showing relationships between environmental samples and reference *Euryarchaea* and *Crenarchaea*, based on 860 aligned bases was constructed according to the LogDet model (Lochkhart et al., 1994) using PAUP version 4.0b software (Swofford, 1999). Bootstrap (1000 replicates) values above 75% are shown at nodes.

### 7.3 Results

To specifically amplify methanogen 16S rDNA, a semi-nested PCR approach was adopted. A single PCR amplification using primer set 1A (forward) and 1404 (reverse) did not produce sufficient amplicon after 35 cycles to be visible by gel electrophoresis (data not shown). Consequently a semi-nested PCR amplification was used, with the primary amplification using 1A and 1404 primer pair and the secondary amplification used primer 1A and the Euryarchaeal specific reverse primer 1100 (reverse). Using this amplification procedure, a product was obtained from all samples (Fig. 7.2). This represented the first successful recovery of methanogen 16S rRNA from copepod faecal pellets.

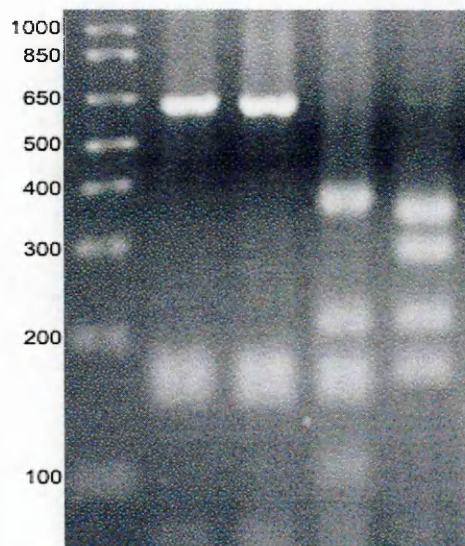


**Figure 7.2** PCR amplification products obtained with semi-nested euryarchaeal-specific primers: Lanes 1&7: 1 kb ladder; Lanes 2-6: replicate PCR products from *T. longicornis* sample; Lanes 8-9; 10-11: Positive and negative controls for the respective 1° and 2° amplification; Lane 12: Positive control solely for the 1° amplification. PCR products were visualized on 1% agarose stained with Ethidium Bromide.

Subsequent to 16S rDNA clone library construction, 40 clones were selected at random and RFLP analysis was performed. The diversity within the clone libraries was limited, with three operational taxonomic units (OTUs) revealed by RFLP analysis (Fig. 7.3).

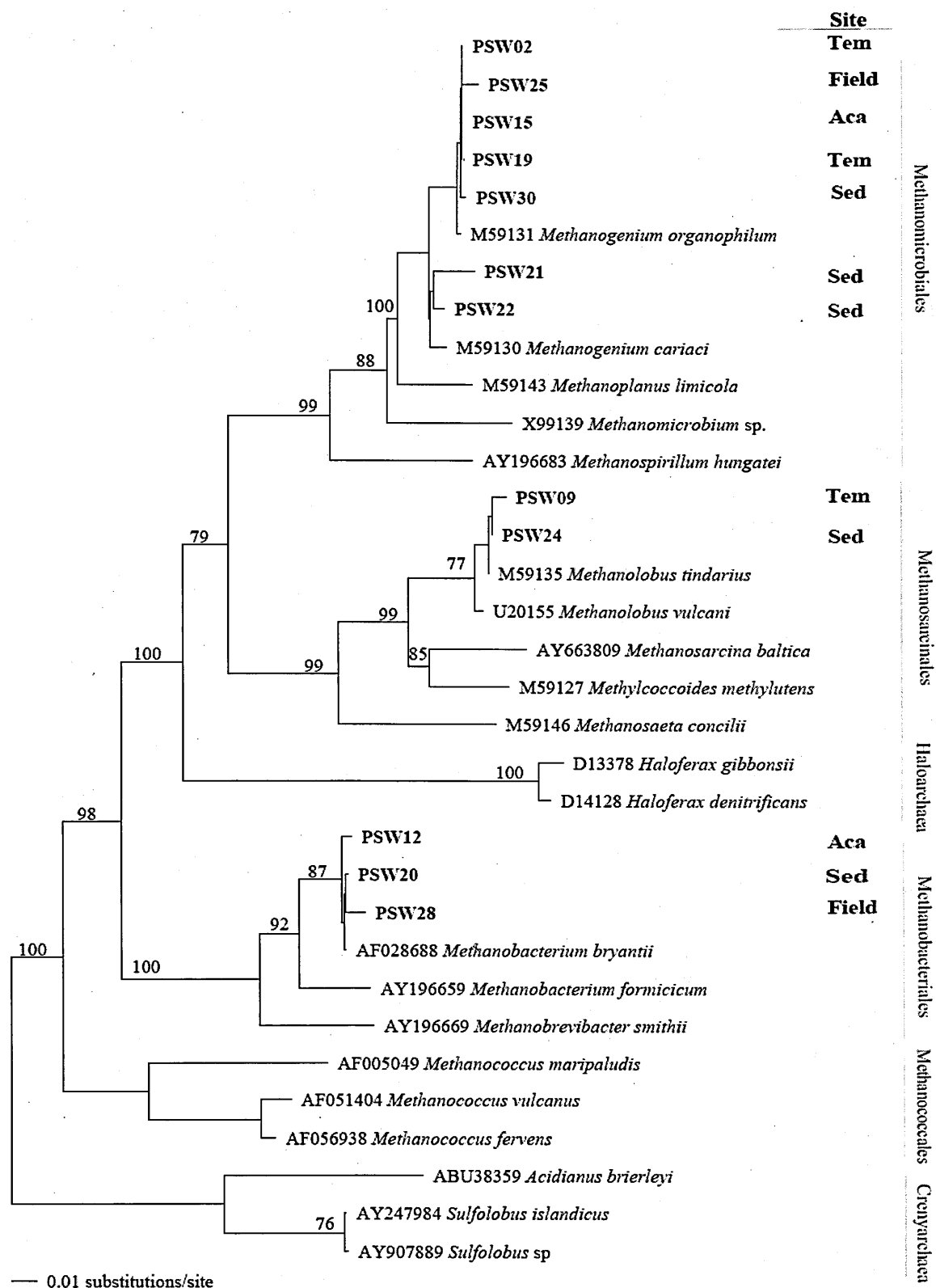
Although the clone library diversity in all samples was limited to these three patterns, there

was evident variation between the different libraries (Table 7.2). In total, twenty-six clones were selected for 16S rDNA sequence analysis.



**Figure 7.3** Gel section showing RFLP analysis of amplification products digested with *RsaI* enzyme: Lane 1: 1kb ladder; Lanes 2-3 *Methanogenium* sequence; Lane 4: *Methanolobus* sequence; Lane 5: *Methanobacterium*. Digested fragments were viewed in 2.5% Metasieve agarose.

Phylogenetic analysis confirmed the presence of three groups as revealed by RFLP analysis. Three distinct clades of 16S Euryarchaea sequences were identified from the cultures and environment. The LogDet/Paralinear distances model used to infer this phylogenetic relationship tree was limited to 860 nucleotides that could be aligned from an original 1100 base-pair DNA product (Fig. 7.4). Bootstrap analysis was performed using 1000 replicates and the confidence of each branch point is shown. Comparative sequences are limited to type strains, although clones related to the sequences are discussed below.



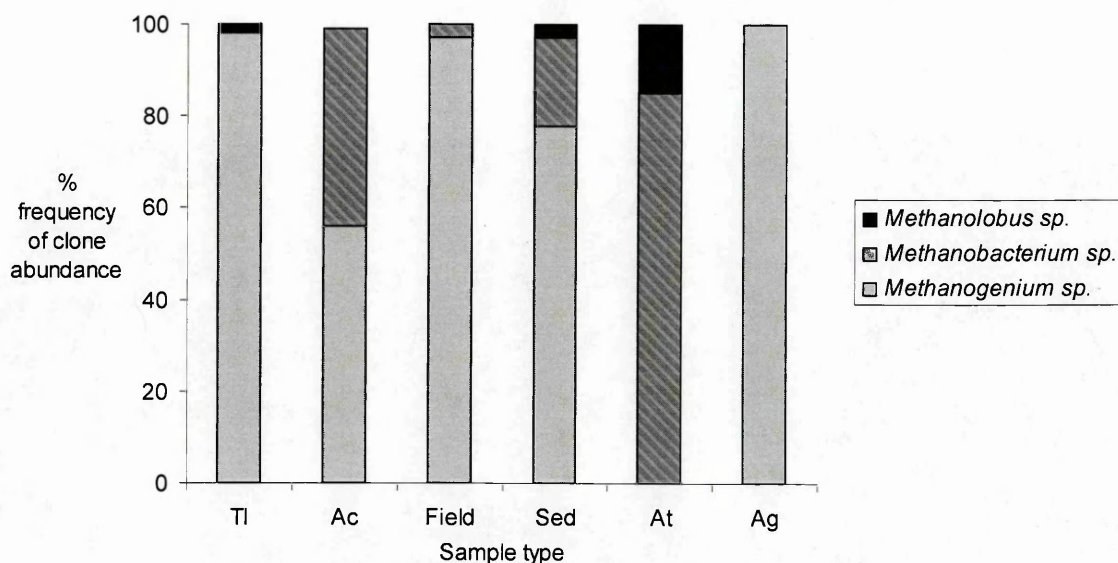
**Figure 7.4** Inferred phylogenetic relationships among archaeal environmental sequences and reference taxa, from 16S rRNA extracted from two copepods *Acartia* (Aca), *Temora* (Tem), field samples (Field) and sediment trap material (Sed). Logdet/paralinear distances tree based on 860 nucleotides that could be aligned. Bootstrap (1,000 replicates) values of 75% are shown.

**Table 7.2** Identification and abundance of methanogen-related clones from Euryarchaeal clone libraries constructed from faecal pellets from *T. longicornis* and *A. clausi* cultures, zooplankton community samples, and from sedimenting particulate material.

Sample and closest identified type strain relative <sup>a</sup>	Nearest relative Accession No.	<sup>a</sup> <sup>b</sup>		Representative Clones	GenBank Accession Number
		Similarity	Clone Abundance		
<b><i>Temora longicornis</i> (Tem)</b>					
<i>Methanogenium organophylum</i>	M59131	99	98	PSW02, PSW19	EF043518, EF043524
<i>Methanolobus vulcani</i>	U20155	96	2	PSW09	EF043523
<b><i>Acartia clausi</i> (Aca)</b>					
<i>Methanogenium organophylum</i>	M59131	99	56	PSW15	EF051587
<i>Methanobacterium bryantii</i>	AF028688	99	43	PSW12	EF051586
<b>Zooplankton field sample (Field)</b>					
<i>Methanogenium organophylum</i>	M59131	99	97	PSW25	EF043528
<i>Methanobacterium bryantii</i>	AF028688	96	3	PSW28	EF043529
<b>Sedimenting material (Sed)</b>					
<i>Methanogenium organophylum</i>	M59131	99	78	PSW30	EF043531
<i>Methanogenium cariaci</i>	M59130	97	(combined)	PSW21, PSW22	EF043526, EF051588
<i>Methanobacterium bryantii</i>	AF028688	98	19	PSW20	EF043525
<i>Methanolobus vulcani</i>	U20155	99	3	PSW24	EF043527
<b><i>Acartia tonsa</i> (Denmark)</b>					
<i>Methanobacterium bryantii</i>	n/a		85	not shown	
<i>Methanolobus vulcani</i>	n/a		15	not shown	
<b><i>Acartia grani</i> (Spain)</b>					
<i>Methanogenium organophylum</i>	n/a		100	not shown	

<sup>a</sup> Clones analysed: *T. longicornis*: 46 clones; *A. clausi*: 40 clones; Zooplankton Field samples: 42 clones; Sedimenting material: 40 clones.

<sup>b</sup> 16S rDNA pairwise similarity to cultured relative. Abundance of clone type in library.



**Figure 7.5** Clone library identity and abundance of methanogen 16S rRNA sequences retrieved from the faecal pellets of TI (*T. longicornis*); Ac (*A. clausi*); Field (*in situ* zooplankton community); Sed (sediment trap material); At (*A. tonsa*) and Ag (*A. grani*).

All three clades belong to well characterised methanogenic genera that are phylogenetically spread between three different methanogenic orders (Methanobacteriales, Methanomicrobiales and Methanosarcinales). Clade I comprised of sequences clustering within the family Methanomicrobiaceae (Class I Methanobacteria; Order I Methanobacteriales) and specifically to the genus *Methanobacterium*. Clade II dominates the clone libraries and is closely affiliated with members of the genus *Methanogenium* within the Family Methanomicrobiaceae (Class II Methanococci; Order II Methanomicrobiales), whilst Clade III contained members of the *Methanolobus*. This genera belongs within the Family Methanosarcinaceae (Class II Methanococci; Order III Methanosarcinales) which contains the methanogens previously identified from marine pelagic material.

### 7.3.1 Clone library analysis

#### *Scottish cultured copepods*

The clone libraries generated from the faecal pellets from *Acartia clausi* and *Temora longicornis* cultures maintained at Dunstaffnage Marine Laboratory were quite different. *T. longicornis* faecal material contained almost exclusively (98% of library, or 45 out of 46 clones) sequences affiliated to the genus *Methanogenium* (e.g. PSW02 and PSW19 Table 7.2; Figure 7.4). High similarity (99%) was shown to cloned sequences from a previous study on methanogenic populations in Antarctic sediments (Purdy et al., 2003), and to the type strain *Methanogenium organophilum* (M59131). A single clone (PSW09) was detected that belonged to the *Methanolobus* genus, with highest similarity (96%) to type strain *Methanolobus vulcani* (U20155) a halophilic sediment isolate (Singh et al., 2005). In contrast, the clone library derived from DNA extracts of *A. clausi* demonstrated that the library was comprised of both *Methanogenium*-like and *Methanobacterium*-like sequences in almost equal fractions (Table 7.2). The *Methanogenium* affiliated sequences (e.g. PSW15) were identical to those within the *T. longicornis* library. Sequences clustering within the *Methanobacterium* (e.g. PSW02) were 99% similar to methanogens detected within termite guts (AB181818) (Deevong et al., 2004) and 98% to the type strain *Methanobacterium bryantii* (M59124).

#### *Field samples*

Similarly, the clone libraries from the environmental samples (faecal pellets and uncharacterized particulate material) showed differences between each other. Copepod faecal material collected from the field sample revealed that as with the *T. longicornis* culture, the clone library was dominated by sequences similar to *Methanogenium*



*organophilum* (PSW25). However, a small number of clones (2 clones out of 42 analysed, represented by PSW28) were closely related to *Methanolobus vulcani*. Analysis of the sediment trap material revealed the greatest diversity of all the clone libraries, with all three methanogenic groups present. Again, sequences related to *Methanogenium organophilum* (99% similar) were the most predominant in the library (e.g. PSW30), comprising of 78% (31 out of 40 clones) of the total, although two clones (PSW21 and 22) were more closely associated to *Methanogenium cariaci* (M59130). Sequences similar to *Mb. bryantii* comprised the next most frequent amplicon within the library (19%) whilst sequences 99% similar to *Ml. vulcani* were comparatively rare and comprised of 3% of the analysed community.

#### *European cultures of Acartia sp.*

Copepod faecal material from the two European laboratories contained sequences relating to the same clusters of methanogens as identified for copepods maintained in culture in Scotland, yet displayed unique clone library diversity. The clone library of *Acartia tonsa*, from Denmark, contained *Methanobacterium* and *Methanolobus*-like sequences. Similar to other clone libraries containing *Methanolobus*-like sequences i.e. *T. longicornis* and particulate material, it is the less abundant sequence. In comparison, the clone library derived from *Acartia grani* faecal material, from Barcelona, contained exclusively sequences relating to *Methanogenium* species. This was the only reported occurrence of a single cluster of methanogens in the faecal pellet samples. It is possible that further analysis of the clone library would have yielded other sequences, such as *Methanolobus*, which is comparatively rarely represented in the clone libraries.



## 7.4 Discussion

### 7.4.1 Successful retrieval of methanogen 16S rDNA sequences

This study successfully retrieved methanogenic archaea 16S rDNA sequences from faecal pellets of two copepods. The recovery of methanogenic sequences in both laboratory and field-derived faecal pellets represents a new discovery. It provides further support to the hypothesis of methanogenesis occurring in particulate material in the oxygenated water column. Micro-habitats such as zooplankton faecal material may facilitate oxygen depletion and therefore stimulate the anaerobic degradation of organic matter. The analysis of faecal pellets collected from monocultures of copepods also proved to be an easily accessible sample material and a good model with which to investigate pelagic methanogens. The faecal pellets are easily enumerable; measurable to obtain size dimensions, and sampling from mono-cultures of copepods identifies the 'host' organism.

It should be noted that to successfully amplify any Euryarchaeal 16S rRNA genes present in the small quantities of sample material, a 70-cycle semi-nested PCR approach was essential. As Euryarchaeal amplicons could only then be identified in our samples, this indicated their comparative rarity compared to bacteria, which could be successfully amplified from the same DNA stock after only 30 cycles (data not shown). A consequence of using so many PCR cycles was the potential of PCR bias and artificial microdiversity (Suzuki and Giovannoni, 1996; Speksnijder et al., 2001) being introduced in to our analyses. To minimise this, for each sample five replicates were amplified and products pooled in-between the primary and secondary amplification procedures. Nevertheless, whilst the clone library analysis must be treated with some caution, it still provides

valuable and highly informative preliminary data about the methanogenic communities present in zooplankton faecal material.

The diversity of methanogen sequences recovered is low compared to other habitats in which methanogen and other Euryarchaea diversity has been studied. Using identical primer sets and semi-nested PCR methodology, almost all methanogenic genera were represented in coastal salt marsh habitat (Munson et al., 1997). In contrast, the diversity within Antarctic sediments, again with identical PCR methodology, demonstrated only limited diversity with 3 tight clusters identified (Purdy et al., 2003). It is clear that the primer sets used have been demonstrated to be able to amplify all known methanogenic groups within the *Euryarchaeota*. It should be noted however that the semi-nested primer 1100A shows mis-matches to members of the dominant planktonic Euryarchaeota groups recently identified within marine pelagic environments denoted as Euryarchaeotal Marine Group II (Massana et al., 2000). This provides an explanation as to why no amplicons were identified to belong to this Marine Group II, although the limited number of full-length 16S rDNA sequences deposited in Genbank means successful amplification cannot be completely discounted.

It is intriguing that no sequences were recovered relating to the previously identified *Methanosarcinaceae* and *Methanococcoides* sp. that were identified in previous studies. One methodological explanation for the variation in the clone libraries obtained in this study compared to van der Maarel (1999) was the use of different primer sets during the PCR amplification process. It is believed that the primers used by van der Maarel (1999) may select the *Methanosarcinales* over other methanogen taxa (as shown by GenBank).

Mis-matches can be found between van der Maarel's primer sequences and the 16S rDNA sequences obtained in this study. This could explain why genera from other methanogen families (*Methanogenium* sp. and *Methanobacterium* sp.) were not detected in their study.

#### 7.4.2 Diversity of methanogen community

It is unclear why the sequences are so tightly constrained within the Methanobacteriales, Methanomicrobiales and Methanosarcinales clusters. RFLP and phylogenetic analysis revealed the presence of at least one of these clusters in all of the samples. The clone library analysis from *T. longicornis* revealed that all but one of the clones affiliated with sequences from a marine sediment isolate *M. organophylum*. A single clone demonstrated high sequence similarity to *M. vulcani*. In contrast, the clone library from *A. clausi* was comprised of two sequence types, affiliated with *M. organophylum* and *M. bryantii*, present in roughly equal abundances. No members of the Methanosarcinales were detected from the *A. clausi* culture, but given the small size of the clone libraries analysed, their presence cannot be discounted.

The comparison between cultured and environmentally-derived faecal pellets demonstrated *Methanogenium*-like sequences again representing the dominant clone, though subtle differences in the rare clone types were evident, with *Mb. bryantii* present within the field faecal library, but no *Methanolobus* affiliated sequences were detected. Sediment trap material was the only clone library that contained all three genera. The greater diversity is not surprising considering that the trap material will contain a diverse array of particulate material including phytodetritus, general particulate organic matter and faecal material from a range of zooplankton and fish species.

The recovery of methanogen 16S rDNA sequences from faecal pellets collected from *Acartia* sp. maintained in culture at other European laboratories is strong supporting evidence that methanogenic archaea are present in copepod-derived faecal material. Furthermore, 16S rDNA sequences were recovered relating to the same methanogen species as identified in samples from Scotland. Clone libraries from *A. tonsa* had both *Methanolobus* and *Methanobacterium*-affiliated sequences, whilst *A. grani* was the only clone library to be comprised of a single *Methanogenium*-like cluster.

At the present moment, it is unclear from these results what environmental pressures or other factors select for these particular methanogen species. The methanogen species may also possess suitable physiological adaptations, such as tolerance to oxygen exposure. Zehnder and Wuhrmann (1977) reported that *Methanobacterium* strain AZ was capable of a higher degree of oxygen tolerance than other species. Similarly, Kiener and Leisinger (1983) discovered considerable variability in the sensitivity of methanogen to oxygen and in particular *Methanosarcina barkeri* maintained viability for over 24 hr upon exposure to air, which was attributed to its forming multicellular clumps. Methanogen species that are more aero-tolerant may also be able to respond quicker to onset of anoxia and therefore outcompete other methanogen species. Alternative adaptations exhibited by methanogens are the interactions with other bacteria, whom may provide metabolic substrate; or facilitate the close proximity to oxygen (discussed further in Chapter 8). It is possible therefore that other methanogens are either outcompeted, or that they have evolutionary lost these physiological adaptations.

### 7.4.3 Implications of methanogen diversity

The diversity of methanogens revealed by this study suggests an alternative metabolic mechanism for pelagic methanogenesis in contrast to current understanding. Within the methanogens, taxonomic grouping is closely related to metabolic types. Until now, all methanogens identified marine pelagic environment were from two genera in the *Methanosarcinaceae* family: *Methanocoides* sp. (Cynar and Yayanos, 1991; van der Maarel et al., 1998) and *Methanosarcina* sp. (Sieburth, 1993). These methanogens, together with *Methanlobus vulcani* identified in this study, and all other members of *Methanosarcinaceae* metabolise methyl compounds, supporting the idea that pelagic methanogenesis can occur via methylotrophy e.g. methylamines and methyl sulfides (Cynar and Yayanos, 1991; Sieburth and Donaghay, 1993). However, for the first time, 16S rDNA sequences relating non-methylotrophic methanogens were also recovered from the pelagic environment. *Methanogenium* and *Methanobacterium* are CO<sub>2</sub>-reducing methanogens, although *Methanobacterium* is limited to H<sub>2</sub> as the electron donor, (Boone et al., 2001) while *Methanogenium* can use H<sub>2</sub>, formate, plus primary and secondary alcohols (Boone et al., 2001). These two genera dominated the clone libraries. These results indicate that the direct metabolism of DMS and DMSP derivatives as discussed in Chapter 6 and Figure 6.10 could have been due to *Methanlobus*-related species. However it is also possible that depending upon the availability of H<sub>2</sub>, formate or other electron donors, *Methanogenium*- and/or *Methanobacterium*-related species were also metabolically active. To understand the activity of methanogen species it is necessary to quantify the activity of the different community species under the appropriate incubation or *in situ* conditions. One possible methodology to investigate methanogen abundance is to use catalyzed

reporter deposition-fluorescence *in situ* hybridisation (CARD-FISH) (Pernthaler et al. 2002).

A consequence of this metabolic pathway is the potential competition with sulphate-reducing bacteria for the same electron donor,  $H_2$ . In sulphate rich environments, sulphate-reducing bacteria typically inhibit  $H_2$ -dependent methanogens by reducing substrate concentrations below the threshold at which methane production is thermodynamically feasible. Such interactions between methanogens, sulphate reducers and also other anaerobic microbes in the pelagic environment are unknown at present, however these results indicate competition does not result in the exclusion of  $CO_2$ -reducing methanogens. These observations raise questions about the dominant metabolic pathway via which methane is produced in the upper ocean; does methylotrophy or  $CO_2$  reduction prevail?

The discovery of methanogen DNA from copepod faecal pellets also raises questions concerning the ability of faecal material to facilitate anoxic processes. Anaerobic processes are known to occur within micro-niches in predominantly aerobic environments (Sieburth, 1988; Peters and Conrad, 1995) and this has also been demonstrated for particles of sediment ranging from as small as 50 to 200  $\mu m$  diameter (Jørgensen, 1977). However, micro-electrode measurements in marine faecal pellets and mass balance theoretical calculations indicate oxygen depletion within pelagic particulate material is limited to specific macro-aggregates ( $>600 \mu m$ ) under specific environmental conditions (Ploug et al., 1997; Simon et al., 2002). The copepod faecal pellets from *T. longicornis* and *A. clausi* measured on average 200  $\mu m$  in length. Therefore any considerations of metabolic activity relating to this study need to consider the sample material is 3 times

smaller than theoretical calculations show possible to host anaerobic processes. Whilst the presence of 16S rDNA sequences does not indicate whether the methanogens are active in copepod faecal pellets, measurements of methane concentrations from other studies indicate their potential to function under suitable conditions (Bianchi et al., 1992; Marty, 1993; Marty et al., 1997). One consideration is that the copepod intestinal tract hosts anaerobic bacteria, as reported for much larger marine invertebrates (McInerney et al., 1995; Preston et al., 1996; Webster et al., 2004) and act as the inoculum for faecal pellet methanogens. The role of meso-zooplankton facilitating anaerobic activity is supported by de Angelis and Lee (1994) who detected methane production from *in situ* cultures of copepods, discussed further in Chapter 8.

## 7.5 Conclusions

Prior this study, two genera within the *Methanosarcinaceae* family had been identified from pelagic particulate material (Cynar and Yayanos, 1992; Sieburth, 1993; van der Maarel et al., 1998). It is now known that the diversity of methanogens extends to other methanogen families within the Euryarchaea. This has been confirmed for faecal material from four species of copepods maintained separately in culture, however it is not known whether this diversity is observed in other cultures of copepods or from samples in the open ocean. To resolve the issue of biological methanogenesis in the oxygenated pelagic environment, an accurate representation of the active methanogen species is considered essential as it will enable the metabolic pathway to be identified; it provides an insight into the structure of the microbial consortia that form an anaerobic food chain; and it also helps to identify the micro-habitats where methanogenesis could occur under suitable conditions. Additional work has been planned to investigate pelagic methanogenesis further. Samples continue to be collected from different species of copepod maintained in culture. In addition, faecal pellets have been collected from natural communities of zooplankton at two geographical regions, the mid-Atlantic and the oligotrophic central North Pacific. It remains to be seen whether the same species or a wider diversity of methanogenic archaea is observed in these samples.





## Chapter 8. Investigating the presence of methanogens in the digestive tracts of copepods

### 8.1 Introduction

The colonization of animal guts by micro-organisms is ubiquitous in nature and relationships between microbes and hosts range from commensal to mutualistic. Through the provision of a relatively homeostatic habitat, the digestive tracts of macro-organisms are important environments in the microbial degradation of organic matter and the biogeochemical cycling of major elements. Digestive tract environments facilitate oxygen depletion through the respiratory activity of facultative and obligate aerobic microbes. Anaerobic conditions are more easily accomplished in larger intestines *e.g.* the 100 litre rumen of bovines, compared to invertebrate guts *e.g.* termites, which are  $10^8$  times smaller (Brune, 1998). As consequences of the size discrepancy, the smaller guts of termites are rarely completely anaerobic and over 40 % of the volume may be aerobic (Brune, 1998). However, termite guts are still a considerable source of methane ( $20 \text{ Tg CH}_4 \text{ yr}^{-1}$ ) to the atmosphere compared to the ruminants ( $80 \text{ Tg CH}_4 \text{ yr}^{-1}$ ) (Fung et al., 1991).

There has been considerable research on the association of methane with the digestive tracts of animals and a survey of over 100 representatives of terrestrial arthropods found methanogenic Archaea in the hindgut of nearly every millipede, cockroach, termite and rose chafer examined from tropical environments (Hackstein and Stumm, 1994). The digestive tracts of termites have particularly been subject to structural, physico-chemical and microbial community analysis, especially with reference to hosting Archaea. The activity of methanogenic Archaea in termites appears to be diet-dependent as  $\text{H}_2$ -

dependent methanogenesis is more frequent in soil-feeding termites, whereas acetogenesis dominates in both lower and higher wood-feeding termites (Brauman et al., 1992).

Molecular 16S rDNA analysis revealed that the Archaeal community within the wood-feeding termites is most closely related to the genus *Methanobrevibacter* (Brauman et al., 2001) and several novel species of methanogens (*Methanobrevibacter* sp.) have also been isolated from the lower termite *Reticulitermes flavipes* (Leadbetter and Breznak, 1996). In comparison, the higher termites, with more varied feeding patterns have more diverse methanogen populations (Ohkuma et al., 1999).

More recently, the molecular characterisation of the microbial community has been linked to the micro-scale conditions associated with the termite digestive tract, using micro-electrode techniques (Brune et al., 1995). Methanogenic activity, pH and H<sub>2</sub> profiles were described for three separate compartments in the higher termite *Cubitermes orthognathus* (Friedrich et al., 2001). The termite digestive tract morphology reflected unique composition of methanogen species with the extremely alkaline anterior hindgut consisting mainly of members of the *Methanosarcinaceae*, whereas *Methanobacteriaceae* and *Methanomicrobiales* predominate in the more posterior compartments (Friedrich et al., 2001).

In contrast to the terrestrial environment, the intestinal biota within marine organisms has been less studied. Research has mainly focused on marine benthic crustacea, such as the deposit feeding shrimp *Neotrypaea californiensis* (Lau et al., 2002) and the hydrothermal vent associated shrimp *Rimicaris exoculata* (Zbinden and Cambon-Bonavita, 2003). Both studies used 16S rDNA sequence analysis to determine the presence of gut microflora that was specific to the host organism and not found in the surrounding seawater. In addition

to benthic organisms, within the pelagic environment, zooplankton are also likely candidates for a gut microflora. It has long been recognised that ecological studies of zooplankton should consider the presence of gut bacteria (Sochard et al., 1979), but there is comparatively little research on this topic. In separate studies, the microflora of the copepods *Temora stylifera* and *Acartia tonsa* were examined (Delille and Razouls, 1994; Hansen and Bech, 1996). Both studies identified a gut microflora which included the bacteria *Pseudomonas* sp. and *Vibrio* sp.. However, gut bacteria do not appear to be ubiquitous throughout the zooplankton and whilst SEM imaging of copepod digestive tracts confirmed the presence of enteric bacteria in *Calanus plumchrus*, no intestinal microflora were found in *Pseudocalanus newmani* (Nagasawa, 1992).

The interest in copepod gut microflora is in part stimulated by the wider issue of organic matter degradation by microbial activity in the pelagic environment. Comprehending the microbial decomposition of organic material is considered a critical factor in order to understand the fate of carbon within the upper water column (Azam and Long, 2001). It has been queried whether bacterial colonisation and degradation of faecal pellets occurs post-egestion or originates from intestinal activity (Gowing and Silver, 1983). An alternative hypothesis is that the bacteria are ingested along with food and not derived from a resident gut population (Nott et al., 1985; Lawrence et al., 1993). The source of bacteria found in copepod faecal material is also an important consideration for anaerobic activity, e.g. methanogenesis, in the pelagic environment. Methanogens have been detected from ocean particulate material (Cynar and Yayanos, 1991; Sieburth, 1993) and more specifically faecal pellets (Chapter 7). However oxygen depletion in particulate material is tightly constrained by the size of material, ambient oxygen levels and bacterial respiration rates (Ploug et al., 1997). Therefore the extent to which methanogenesis can

occur *in situ* within small particulate material within the pelagic environment remains uncertain. One possible solution that has been widely advocated is the potential for biological methanogenesis in the digestive tracts of zooplankton.

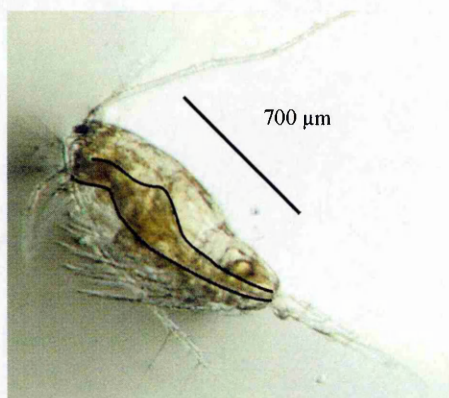
A direct association between methanogens and zooplankton is supported by previous observations of methane production during incubations of copepods. In a comprehensive study, de Angelis and Lee (1994) measured methane production rates from 4 to 20 nmol CH<sub>4</sub> copepod<sup>-1</sup> d<sup>-1</sup> with three species of copepods *Acartia clausi*, *Temora lonicornis* and *Calanus pacificus* when fed either *Thalassiosira weissflogii*, *Rhodomonas lens* or *Prorocentrum minimum*. The authors concluded that the digestive tracts were the most likely site for biological methanogenesis. On two other separate occasions, anaerobic incubations of copepod samples collected from the marine environment produced methane. Bianchi et al., (1992) detected 0.10 to 0.25 µmol CH<sub>4</sub> dm<sup>-3</sup> day<sup>-1</sup> in anaerobic media inoculated with copepod samples collected from the Mediterranean. Similarly, Oremland (1979) measured an increase in methane of 4 orders of magnitude in copepod cultures and subsequently used the addition of methanogen inhibitors to identify that biological methanogenesis was responsible. These observations suggest that methanogenic Archaea are present and active in the digestive tracts of copepods. This opinion is supported by other researchers who investigated the distribution of methane in the upper ocean, such as Traganza et al., (1979) who found a clear correlation between zooplankton ATP biomass and methane concentrations in the East Atlantic. In addition, Tilbrook and Karl (1994) concluded that the most likely explanation for the observed methanogenesis in particles was that it would occur in the guts of organisms and immediately after defecation.

This study investigated the presence of methanogenic archaea in the digestive tracts of *Temora longicornis* and *Acartia clausi* copepods. The same DNA amplification procedure used to identify methanogenic Archaea in copepod faecal pellets (Chapter 7) was adopted.

## 8.2 Method

### 8.2.1 Sample collection

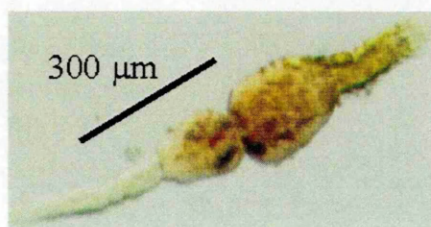
The copepods *T. longicornis* and *A. clausi* were kept in culture as discussed in Chapter 7. The copepods were maintained on a diet of *Rhinomonas* sp. and *Oxyhrris marina* and adult copepods were collected for sampling approximately three hours after feeding. Therefore samples represent full-gut copepods, distinct from voided-gut individuals. The copepods were rinsed in sterilised seawater, transferred to a glass Petri dish and measurements of copepod body sizes were made using a stereomicroscope (Figure 8.1).



**Figure 8.1** *Acartia clausi* copepod with digestive tract outlined.

To dissect the gut, the copepods were rendered motionless by the removal of the seawater medium using a micro-pipette, with no anaesthesia required. Fine-tipped forceps were used to hold the copepod by the upper cephalosome, whilst the lower urosome was very gently pulled to remove the entire digestive tract (between the oesophagus and anal aperture) in one piece (Figure 8.2). Any non-intact or damaged digestive tracts were discarded. For both species of copepod, the sampling procedure was repeated for groups

of 20 and 80 adult individuals, providing a total of four samples for the DNA extraction procedure. The digestive tracts were transferred by glass micro-pipette to a 1.5 ml collecting tube. Samples were centrifuged at 12,000 x g for 10 min and stored at -80 °C.



**Figure 8.2** Dissected gut (intact) from *A. clausi*.

### 8.2.2 DNA extraction

DNA was extracted following the procedure of Henckel (1999) (Table 2.1) with a few modifications. Approximately 0.5 g zirconia-silica beads (0.1 mm diameter) were added to the samples with 250  $\mu$ l NaPh buffer (120 mM pH 8.0) and 80  $\mu$ l SDS solution (10% SDS; 0.5 M Tris-HCl pH 8.0; 0.1 M NaCl). Samples were vortexed and cells lysed with cell disruptor (Qiagen) at 20 Hz for 1 min. The cell lysis step was repeated twice with samples kept on ice for 1 min between the lysis steps. Samples were subsequently centrifuged for at 12,000 x g for 3 min. The supernatant was collected and resuspended in 400  $\mu$ l phosphate buffer. Protein and debris were precipitated from the supernatant by adding 0.4 vol of 7.5 M ammonium acetate. Samples were then incubated on ice for 5 min and centrifuged at 12,000 x g for 4 min. Nucleic acids were precipitated with 0.7 volume isopropanol and then centrifuged at 12,000 x g for 45 min at 4°C. The resulting DNA pellet was washed with 100  $\mu$ l 70% ethanol at 4°C and dried under vacuum. DNA was re-suspended in 100  $\mu$ l TE (10 mM Tris, 0.1 mM EDTA, pH 8.0) buffer before storage at -80°C.



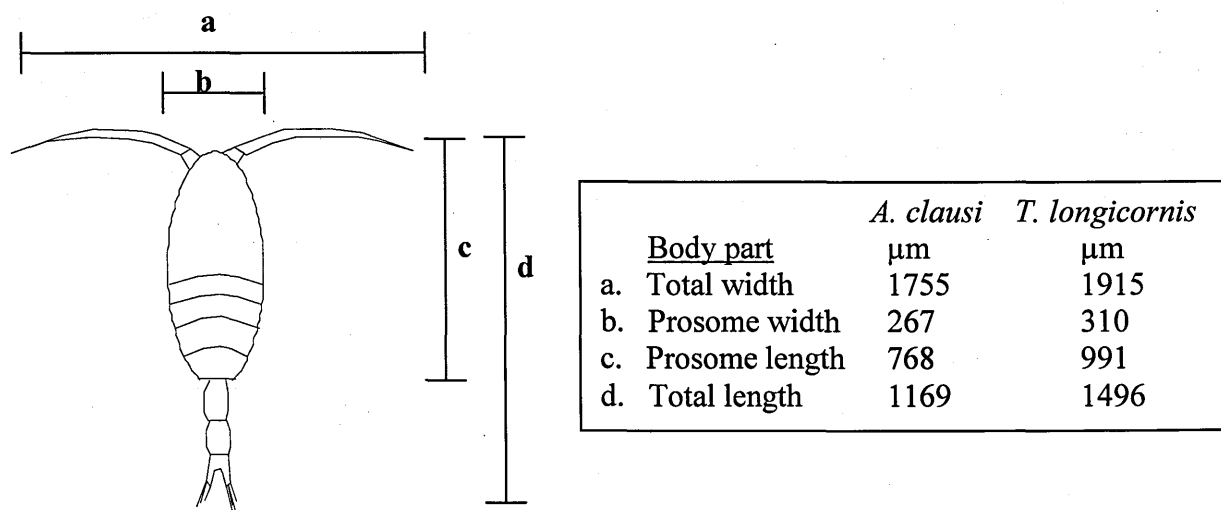
### 8.2.3 Amplification of 16S rDNA

To confirm a successful extraction of DNA and investigate the presence of other prokaryotes, PCR was performed using the universal bacterial primers 27F and 1492R (Lane, 1990). 2 µl of DNA template was added to a PCR mixture (50 µl) containing 5 µl buffer, 4 µl MgCl<sub>2</sub> (25 mM), 2 µl of forward 27f and reverse 1492R primer (10 pmol), 2 µl bovine serine albumin (100 µg/ml), 0.5 µl dNTP (20 mM), 1 U *Taq* polymerase (AB gene, UK) and 34.3 µl H<sub>2</sub>O. PCR amplification involved denaturing at 95 °C for 5 min, followed by 28 cycles (55 °C for 30 sec, 72 °C for 3 min, 94 °C for 10 sec) then heating at 55 °C for 30 secs with a final extension of 72 °C for 10 min followed by cooling at 10 °C for 1 min (PTC 200). *Methanospirillum* plasmid was used as a positive control, whilst no DNA was added in the negative control. PCR products were viewed on 0.6% agarose gel by electrophoresis stained with Ethidium Bromide.

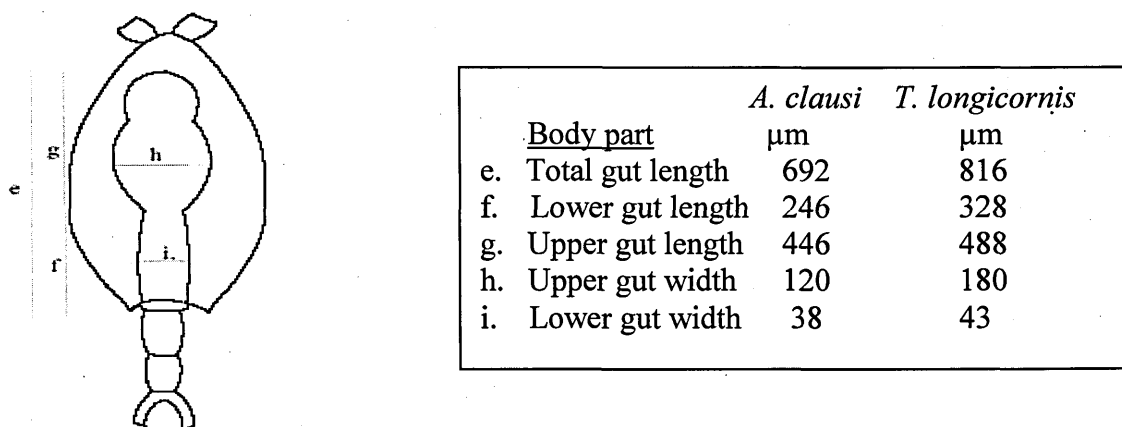
Euryarchaeal 16S rRNA gene sequences were amplified using the hemi-nested procedure described by Chapter 7. In brief, each PCR procedure was performed on five replicates samples to avoid stochastic biases (Wagner et al., 1994) using *Taq* polymerase (AB gene, UK). PCR products from the primary amplification (1Af and 1404R) (Embley et al., 1992) were pooled, concentrated to 20 µl with MicroCon 30 cartridges (Millipore, UK) and then five replicate reactions using 2 µl aliquots of the primary PCR product were amplified in the second round of PCR using the semi-nested 1Af and 1100R primer set. Positive controls consisted of *Methanospirillum* and negative controls consisted of no DNA addition.

### 8.3 Results

The body sizes of the copepods (Fig. 7.3) and their digestive tracts (Fig. 7.4) were measured for both species. *T. longicornis* is the larger individual measuring 1.5 mm in length compared to *A. clausi* (1.1 mm). Additional images of *A. clausi* gut morphology are present in (Hassett and Blades-Eckelbarger, 1995) and also (Arnaud et al., 1980).

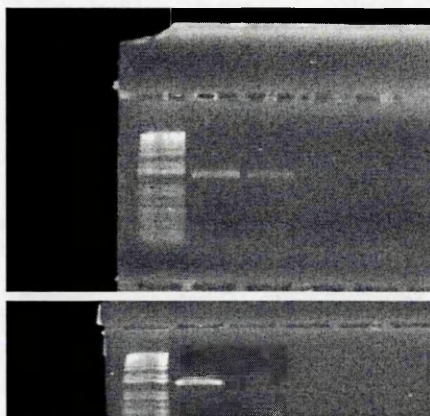


**Figure 7.3** Schematic diagram to show how the size characteristics of copepods were measured.



**Figure 7.4** Schematic diagram to show gut measurements were taken

A DNA product with the bacterial primers demonstrated DNA extraction was successful for all samples of both species of copepod. Figure 7.5 shows the bacterial 16S rDNA products obtained with universal bacterial primers for *A. clausi* digestive tract samples. Furthermore, a clear difference was observed in the quantity of DNA retrieved from guts of 20 and 80 copepods. A similar result was obtained for *T. longicornis* though the data is not shown.



**Figure 7.5** Top: Bacterial 16S rDNA product obtained with 27f and 1492 primers. Lane 1: 1 kb ladder; Lane 2: 80 x *Acartia clausi* guts; Lane 3: 20 x *Acartia clausi* guts. **Figure 7.5b** (bottom): Lane 1: 1 kb ladder; Lane 2: Positive control of *Methanospirillum* sp. plasmid; Lane 3: Negative control; Lane 3: Absence of a Euryarchaeal PCR product.

The presence of methanogenic archaea was investigated using the universal euryarchaeal 1Af and 1404/1100R primer combination. Amplification with this primer set had previously yielded PCR products from *Methanogenium*, *Methanlobus* and *Methanobacterium* (Chapter 7). Amplification of the positive control *Methanospirillum* also yielded a PCR product in all procedures (data not shown). However, no amplification product could be detected when different quantities (0.1 – 5.0 µl) of DNA template from the digestive tract were used. This suggests that either no methanogenic archaea were present or that they were beneath the detection limit. This contrasts with the PCR product

obtained using the 27f and 1492R universal bacterial primers (Fig. 7.5) indicating that bacteria were present in the digestive tracts.

## 8.4 Discussion

This research investigated the presence of methanogenic archaea in the digestive tracts of two commonly occurring calanoid copepods, *Acartia clausi* and *Temora longicornis*.

Although the DNA extraction process was successful, 16S rRNA gene analysis did not detect any Euryarchaeal sequences. The negative result can be interpreted two ways: either methodologically-influenced whereby methanogenic Archaea were present, but beneath the level of detection or alternatively, methanogenic Archaea are genuinely absent which reflect the physiological conditions of copepod digestive tracts. These possibilities are discussed, with reference to the method protocol and copepod physiology.

### 8.4.1 Analytical procedure

The absence of Euryarchaeal 16S rDNA sequences in copepods digestive tracts contrasts with the identification of methanogenic 16S rDNA in faecal pellets from the same copepod species (Chapter 7). Considering that copepod gut contents are voided as faecal material, this represents an enigmatic result. One issue to consider is whether the quantity of sample volume is comparable between the digestive tract and the faecal pellets. Comparison of sample volumes reveals sample material from 80 individuals of *Acartia clausi* and *Temora longicornis* digestive tracts represents 5 times and 12 times respectively the quantity of faecal material previously analysed (Chapter 7). This is calculated assuming that all of the gut contents are excreted as faecal material, a cylindrical volume for digestive tracts, with a density of 1.1 g per cm<sup>3</sup> of material (Butler and Dam, 1994; Feinberg and Dam, 1999).

If it is assumed that a PCR product should have been obtained *i.e.* methanogenic Archaea were beneath detection limit (even after 70 cycles of semi-nested PCR), there are a number of methodological explanations for the negative result from gut material. One possibility is the different nucleic acid extraction protocols that were used for faecal pellets (chemical extraction) and for digestive tracts (mechanical extraction). However, although the loss of nucleic acid is inevitable during any extraction protocol, there is no evidence that increased DNA is lost during the mechanical extraction procedure. Similarly, humic acid contamination is one of the biggest problems in molecular microbial ecology (Purdy, 2005), however there is no indication that it was a greater problem in the gut samples as a PCR product was obtained with a bacterial primer set (Fig. 7.5). Perhaps the most plausible explanation lies in the nature of the sample material as it may be difficult to extract DNA from small quantities ( $\leq 1$  mg) of gut material which require a mechanical extraction method, such as the bead-beating procedure used in this study and elsewhere (Henckel et al., 1999). Ironically, the lack of a Euryarchaeal 16S rDNA product in copepod samples compared to faecal material contrasts to previous research on anaerobic purple-sulfur bacteria in copepod samples (Proctor, 1997). Proctor (1997) detected the anaerobic bacteria in copepod samples, but not faecal material. Although a bacterial PCR product was obtained from the both copepod samples in this study, the wider bacterial community was not characterised and it is not known whether the purple-sulfur bacteria as identified by Proctor (1997) are present in our samples.

#### **8.4.2 Absence of methanogenic archaea**

The alternate explanation to the absence of a Euryarchaeal PCR product is that this reflects the true scenario and it can therefore be reported that there is no resident population of

methanogens in copepods. The absence of any methanogens is a likely consequence of the digestive tract physiology associated with the copepods, as reflected by the three factors of size; structure; and gut passage time.

### Size

In comparison with other digestive tracts, the guts of copepods represent particularly small digestive tract environments. The volume of the upper gut for the copepods (*Acartia clausi*:  $5 \times 10^6 \mu\text{m}^3$ ; *Temora longicornis*:  $12 \times 10^6 \mu\text{m}^3$ ) is  $10^3$  times smaller than the more commonly studied hindgut of the termite ( $1 \times 10^9 \mu\text{m}^3$ ). A consequence of the small volume is a large surface area to volume ratio which will increase the aerobic capacity of the gut environment. Oxygen concentrations have not been reported for copepod guts, although macro-injection techniques, similar to those routinely used in termite work (Brune et al., 1995), have been used to measure pH in copepod digestive tracts (Pond et al., 1995). However, there must be a lower size limit below which digestive tract geometry cannot support anoxia at any given removal rate for oxygen, as reported for particulate material (Ploug et al., 1997). Based on the measurements of the copepod upper gut section, an oxygen penetration depth of 55  $\mu\text{m}$  for *A. clausi* and 90  $\mu\text{m}$  for *T. longicornis* would make the gut entirely aerobic. These values are at the lower end of the range (50-250  $\mu\text{m}$ ) reported for oxygen penetration into termite guts embedded in agarose (Brune, 1998).

### Structure

The digestive tract structure plays an important role in gut microflora. This is apparent in the guts of termites where the different gut compartments have specific chemical conditions and specific Archaeal community (Schmitt-Wagner and Brune, 1999; Friedrich

et al., 2001). Of all the gut regions, the hind gut is the most amenable to hosting an indigenous microbiota (Plante et al., 1990; Harris, 1994). In the hindgut, the main function is the storing faecal material and is the area where the host's defences against microbes (e.g. enzymes, surfactants and sloughing) are weakest. Additionally, bacteria in the hindgut have access to leftover material undigested from passage through the midgut and are therefore not competing directly with their hosts for uptake of digested compounds. However, neither species of copepod studied have a characterised hindgut (Arnaud et al., 1980). This is in contrast to larger calanoid species, e.g. *Calanus helgolandicus* (Pond et al., 1995). Food is transferred via the oesophagus to the anterior region of the midgut where it accumulates for <20 minutes before passing to the posterior region of the midgut where the faecal pellet is formed for subsequent defecation (Mauchline, 1998).

#### *Gut passage time*

A consequence of the small body size and digestive tract is a short gut passage time. The length of time from digestion to excretion is usually under one hour (Mauchline, 1998) and may be as little as 20 minutes (Dam and Peterson, 1988). This is extremely quick in comparison to other organisms such as the soil-feeding termite *Proculitermes aburiensis* which is ~10 times larger than *A. clausi*, yet gut passage time is 36 to 48 hours (Bignell et al., 1980). Therefore any resident enteric gut microflora will have little time to become established.

The three factors of digestive tract size, structure and passage time highlight the constraints of the digestive tract to facilitate oxygen depletion and host an anaerobic microbiota. Based on size constraints alone, it is possible that anoxia may occur, particularly in *Temora longicornis*. However, in combination with the other gut

characteristics of the quick gut passage time and gut structure, the evidence suggests that digested material will be excreted with limited oxygen depletion which continues inside the faecal pellet.

#### 8.4 Conclusion

It has been hypothesised that copepod guts represent micro-environments which are more likely to facilitate oxygen depletion than faecal material. The larger size of copepod guts in comparison to faecal material has been used as a possible explanation for the discrepancy between methanogenesis in particulate material with the constraints on oxygen depletion. This work investigated the digestive tract of two commonly occurring copepods for methanogenic archaea using 16S rDNA analysis. The absence of a DNA product after two rounds of semi-nested PCR amplification suggests that copepod digestive tracts may not facilitate methanogenesis as previously anticipated. The interpretation of the negative results is presented from both a methodological and physiological perspective. On consideration of both interpretations it is thought most likely that numbers of methanogens were beneath the level of detection using this particular methodology. However consideration of the copepod gut suggests that anaerobic conditions within the gut are equally constrained as the faecal pellets themselves and it is unlikely that the oceanic methane paradox can not simply be resolved by proposing a larger micro-environment which could facilitate oxygen depletion. It is suggested that future work should focus on the presence of a wider anaerobic food web or methanogen physiology to resolve the presence of methanogens in particulate material within the upper water column.





## Chapter 9. Discussion

This thesis investigated methylated sulphur and methane biogeochemistry within microhabitats in the upper water column. A methodological approach was adopted which investigated DMSP, DMSO and DMS in the upper water column and looked at the utilisation of these compounds by the methanogenic Archaea. This investigative route revealed a number of interesting scientific aspects. The production of DMSO by major phylogenetic groups was characterised. Concentrations of DMSO were found to be taxon-dependent and represented ~20 % of total intracellular methylated sulphur (DMSP and DMSO). The fate of algal-DMSP and DMSO was analysed in a series of laboratory-grazing experiments. These laboratory-based experiments were complimented with a field-based survey of the sedimentation of DMSP and DMSO and changes during the sinking process. Incubations of field-collected material revealed that a net production of DMSO was observed in these particles. The incubation of particulate material also revealed the production of methane. The addition of DMSP, DMSO and DMS to the samples stimulated a greater increase in methane concentrations, whilst the addition of inhibitors revealed the presence of a wider anaerobic microbial consortium that could facilitate the degradation of these algal-compounds. The methanogen community was characterised using 16S rDNA analysis and sequences relating to both methylotrophic and CO<sub>2</sub>-reducing methanogens were discovered. This suggests that the methylotrophy is not the only metabolic pathway by which methane is produced in the upper water column.

To review these findings and relate the work to the wider context of ecosystem functioning, this Chapter is split into three sections. The first section deals with the micro-habitat and the potential for anaerobic metabolism within the pelagic environment. The

second section looks at methane and DMS biogeochemistry on an oceanic scale, by comparing data collected as part of the Atlantic Meridional Transect programme. The final section considers how the physiology and metabolism of the micro-organisms can affect globally important processes through biogenic gas formation.

### **9.1 Micro-niches and anaerobic metabolism in the pelagic environment**

In the marine environment, the production of the methylated sulphur compounds DMSP and DMSO is associated with phytoplankton, as demonstrated in Chapter 3. Once released to the ambient environment, DMSP and DMSO are a potential source of carbon and sulphur for bacteria. The microbial-mediated cycling of DMSP and DMSO also plays a key role in the production of DMS. The emission of DMS to the atmosphere and the subsequent oxidation products affect the Earth's radiative forcing, by cooling the Earth's climate (Section 1.1.4). However DMSP, DMSO and DMS also represent metabolic substrates for methanogenic Archaea, either indirectly or directly. Within micro-environments in the upper water column, bacterial respiration can cause localised oxygen depletion which facilitate methanogenesis and the wider anaerobic degradation of organic matter. The implication of this additional pathway is that methane, a potent greenhouse gas, can be produced from compounds that are typically considered to be precursors to atmospheric aerosols.

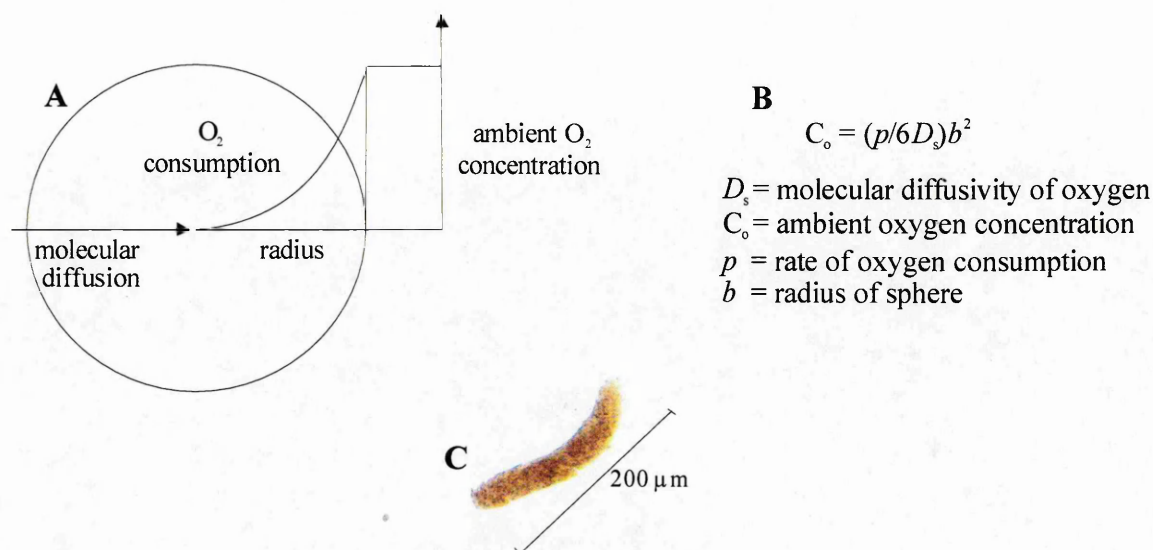
A critical step in this process appears to be the repackaging of algal cell constituents into particulate material. This can occur through the release of algal cell material into solution and the subsequent growth of aggregates (Alldredge et al., 1993). One of the first reports on particulate organic material in the marine environment looked at the formation of organic aggregates from dissolved organics derived from diatom cultures (Riley, 1963).

Particulate material can also occur due to the grazing and ingestion of algal cells, with subsequent excretion of faecal pellets by zooplankton (Chapter 4). There has been increased focus on particulate material, including aggregates and faecal pellets, as researchers try to understand the marine microbial activity within the upper ocean (Azam and Worden, 2004). Azam (1998) highlights that particulate material can support elevated microbial diversity and metabolic activity in the upper water column. Furthermore, the downwards flux of particulate material and subsequent burial in the sediments represents a sink for atmospheric CO<sub>2</sub> (Wollast, 1991). Therefore an understanding of whether the oceans will act as a source or sink for carbon in future climate change scenarios depends on whether we can comprehend the dynamics of aggregates and faecal pellets.

Particulate material can also play an important role in facilitating the production of trace gases. Copepod faecal pellets were identified as DMSP 'hotspots' in Chapter 4, in comparison with ambient DMSPd concentrations. If bacterial sulphur demand is satiated in these micro-environments, then increased production of DMS production via bacteria-mediated DMSP cleavage could occur (Kiene et al., 2000; Scarratt et al., 2000). Furthermore, any change in the redox potential gradients within the particle could influence the microbial transformation of DMSO (Lee et al., 2004). Suspended particulate material also offers the possibility for the innermost part of the particle to become anaerobic. There are increasing reports of obligate anaerobes being isolated from the pelagic environment (Cynar and Yayanos, 1991; Sieburth, 1993), and the identification of facultative anaerobic metabolism (Riemann and Azam, 2002; Alonso and Pernthaler, 2005), which suggest that marine bacteria might encounter anoxic microzones more frequently than would otherwise be suspected in the predominantly aerobic pelagic environment. In Chapter 6, the detection of methane in particulate material collected from

the pelagic environment and incubated under anaerobic conditions also indicates the potential for *in situ* methanogenesis under the appropriate conditions. This is supported by the identification of methanogenic Archaea in copepod faecal pellets using 16S rDNA analysis in Chapter 7.

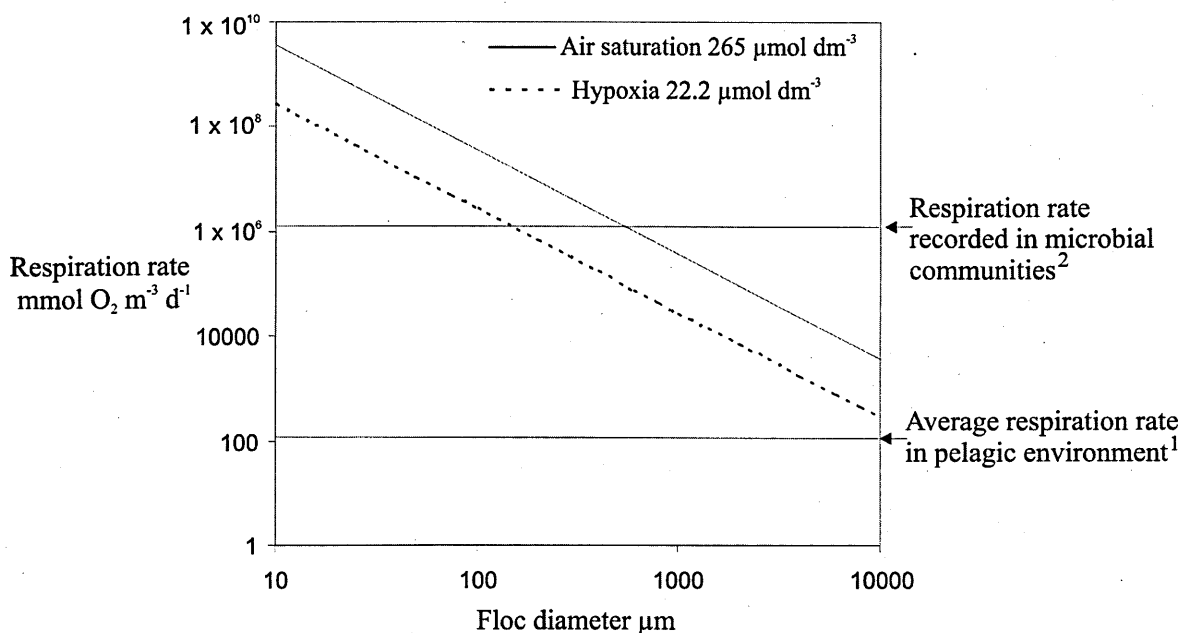
However, neither the incubation experiment, nor the characterisation of the methanogen community by 16S rDNA reveals the *in situ* activity of methanogens within copepod faecal pellets in the surface oceans. The potential for anoxia to occur in copepod faecal pellets, or any particle and aggregate, is influenced by chemical, physical and biological constraints. These constraints include ambient oxygen concentrations, molecular diffusion of oxygen, the size of the particle and oxygen consumption rates by bacteria (Jørgensen, 1977) (Fig. 9.1). These parameters have been used to calculate the potential for oxygen depletion in a range of environments including anoxic aggregates (Tilbrook and Karl, 1994; Ploug et al., 1997) aerated sewage sludge (Schramm et al., 1999) and cyanobacteria aggregates (Tuomainen et al., 2003). The calculation can also be applied to the faecal pellets of *Acartia clausi* and *Temora longicornis* assuming: an ambient oxygen concentrations ( $265 \mu\text{mol O}_2 \text{ dm}^{-3}$ ); the faecal pellet radius of  $40 \mu\text{m}$ ; and molecular diffusion is assumed to be  $2.4 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$  (Jørgensen, 1977).



**Figure 9.1** (A) The distribution of oxygen in a spherical particle as produced by the interaction between oxygen influx and removal. (B) The associated model equation is taken from Tilbrook and Karl (1994), after Jørgensen (1977). (C) A faecal pellet from *Acartia clausi* copepod.

Under these conditions the centre of the particle would become anaerobic at respiration rates  $\geq 0.0191 \mu\text{mol } O_2 \text{ cm}^{-3} \text{ s}^{-1}$  (equivalent to  $1.65 \times 10^6 \text{ mmol } O_2 \text{ m}^{-3} \text{ d}^{-1}$ ). These rates of respiration exceed the average respiration rates typically observed in the pelagic environment by 4 orders of magnitude (Robinson and Williams, 2005) and also the volumetric rates of respiration observed in other microbial communities (Schramm et al., 1999). It can therefore be assumed that anaerobic degradation of organic matter is unlikely to occur in the small faecal pellets of meso-zooplankton, as similarly concluded by other researchers (Jørgensen, 1977; Alldredge and Cohen, 1987; Tilbrook and Karl, 1995; Ploug et al., 1997). This creates a dilemma in accounting for the presence of obligate anaerobic microbes and the occurrence of reduced gases in the oxygenated environment. A number of possible explanations can be inferred to account for this discrepancy:

i) The effect of increasing particle size and decreasing ambient oxygen concentration combine to decrease the respiration rate required for oxygen depletion (Fig. 9.2). A theoretical minimum size limit of ~500 to 700  $\mu\text{m}$  under oxygen saturation conditions is suggested by Figure 9.2, which is similar to previously calculated size of ~600  $\mu\text{m}$  required for oxygen depletion (Simon et al., 2002). This suggests that the aggregation of faecal pellets at density gradients within the water column, *e.g.* the pycnocline, may increase the likelihood for anoxia to occur within the particles. Figure 9.2 also highlights that oxygen depletion is more likely to occur under low ambient oxygen pressure.



**Figure 9.2** Volumetric oxygen respiration rates required to create anoxia at the centre of a particle under different ambient oxygen concentrations, air saturation and hypoxic conditions. The horizontal dotted lines indicate microbial respiratory rates reported in the literature <sup>1</sup>Robinson & Williams (2005); <sup>2</sup> Schramm (1999).

One part of the water column where concentrations of oxygen could alter is density gradients where 'planktonic thin layers' can form. These consist of phytoplankton,

zooplankton and particulate material and have been observed in coastal environments (Alldredge et al., 2002; McManus et al., 2005) and open ocean waters (Bjornsen and Nielsen, 1991; Gorsky et al., 2003). These planktonic layers are typically associated with physical discontinuities in the water column such as the base of the pycnocline (McManus et al., 2005). Advances in optical and acoustical sampling (Cowles and Desidero, 1993; Holliday et al., 1998; Doubell et al., 2006) have revealed that these layers range from a few centimetres to a few metres thick and can extend up to many kilometres horizontally.

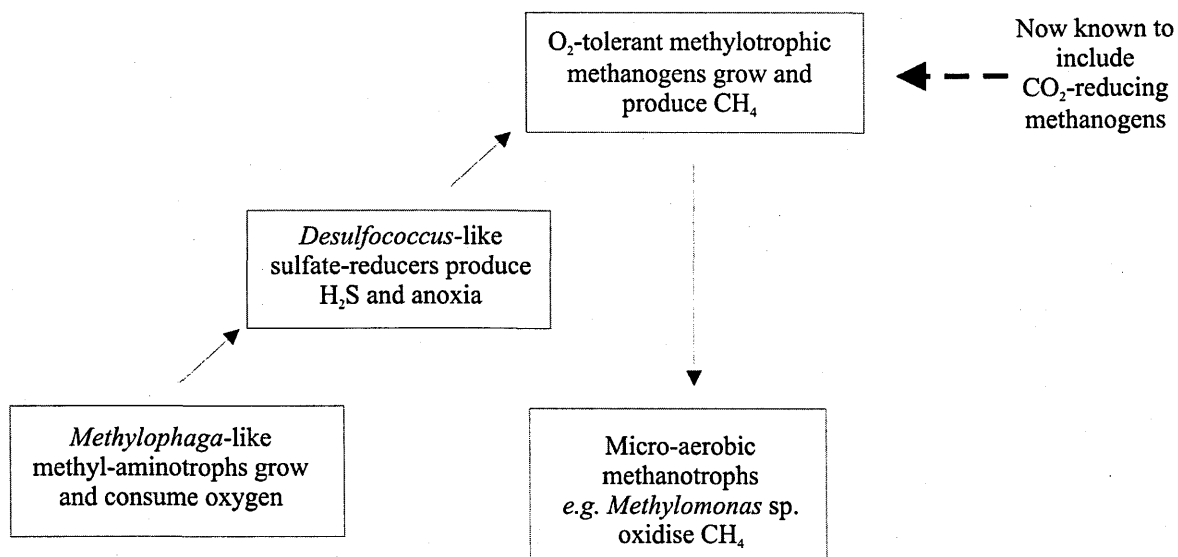
ii) Copepod faecal pellets are membrane-covered surrounded by a peritrophic membrane that is also found in the faecal material of other crustaceans. The peritrophic membrane appears to consist of chitinous microfibrils, polysaccharides and proteins (Frangoulis et al., 2004). Whilst the role of this membrane is debated and appears to have many different functions, it may also limit oxygen diffusion inwards and therefore facilitate localised anoxia. One technique used to examine micro-habitats and boundary layers are micro-electrode sensors. These can be progressively moved through the bio-layer of different habitats *e.g.* biofilms, sediments, particulate material (Ploug et al., 1997; Schramm et al., 1999) and the digestive tracts of invertebrates, to record the physico-chemical gradients *e.g.* O<sub>2</sub>, pH, sulphide, CO<sub>2</sub> and N<sub>2</sub>O (Revsbech, 2005). Combined with phylogenetic analysis, micro-electrode studies provide direct spatial measurements of the proximity of methanogenic Archaea to oxygenated environments. Analysis of termite gut environments revealed methanogens located between 200 to 400 µm from ambient oxygen (Brune et al., 1995), whilst in anaerobic aggregates methanogens have been found situated 100 µm from the surface (Santegoeds et al., 1999). It is possible that the spatial resolution of micro-electrodes may be challenged for a 40 µm faecal pellet, however Ploug (1997) suggests



that with a tip diameter of 2-6  $\mu\text{m}$  and a response time of 0.2 sec, anoxic microzones would be detected.

iii) Methanogens could also be present in copepod digestive tracts, with subsequent inoculation of the faecal pellets. This was proposed by de Angelis and Lee (1994) after observing the production of methane during grazing by copepods. However, no methanogen 16S rDNA sequences were detected in the digestive tracts dissected from *A. clausi* and *T. longicornis* in Chapter 8. These results indicate that methanogens are not resident in digestive tracts, but are ingested with the food and excreted in faecal pellets.

iv) It is also possible that methanogens have cellular physiological adaptations for life in the pelagic environment. Kiener and Leisinger (1983) identified that certain methanogen species (*e.g. Methanosarcina barkeri*) maintained viability for over 24 hours after exposure to air. More recently, various anti-oxidant enzymes include catalases and superoxide dismutases, which provide defence against the toxic effects of products resulting from the incomplete reduction of oxygen have been identified in methanogens including *Methanobacterium bryantii* (Brioukhanov and Netrusov, 2004). In addition, the interactions between methanogens and other anaerobic microbes may be important as a wider microbial consortium including sulphate-reducing bacteria, fermentative bacteria and methane oxidisers is considered essential for substrate supply and oxygen depletion (Megonigal et al., 2004). Sieburth in particular has been a strong advocate for a methanogenic bacterial consortium (Sieburth, 1988; Sieburth, 1991; Sieburth and Donaghay, 1993) (Fig. 9.3).



**Figure 9.3** Diagram illustrating a microbial consortia that are hypothesised to facilitate methanogenesis in the upper ocean (Taken from Sieburth 1991). A possible sequence of events is the methylaminotrophs deplete oxygen and produce formate for the sulphate-reducing bacteria, whose hydrogen-sulfide creates the strictly anaerobic conditions for the growth of methanogens.

### *Fermentative bacteria*

The increase of methane concentrations with the addition of DMSP as observed in Chapter 6 highlights the importance of fermentation in the degradation of high molecular weight compounds as methanogens are unable to directly metabolise the sulphur compound. Previously, the methylotrophs *Methylophaga* sp. has been isolated from the pelagic environment (Sieburth et al., 1987). It remains to be seen whether specific relationships exist between fermenting bacteria and methanogenic Archaea in the micro-niches. A syntrophic relationship was proposed for the methanogen population and *Syntrophomonas* identified in anaerobic aggregates. (Santegoeds et al., 1999). Interestingly, certain species

of *Syntrophomonas* are capable of using DMSO as an electron acceptor and it is possible that the methanogens can utilise the DMS produced.

### *Sulphate-reducing bacteria*

During the anaerobic incubation of particulate material collected from the pelagic environment, both sulphate-reducers and methanogens were active in the degradation of DMSP and DMS (Chapter 6). Furthermore, it was hypothesised that methanogens utilised the metabolic end-products of sulphate reduction in the presence of DMSP, which suggests that under certain conditions sulphate-reducers can facilitate methanogenesis. The co-existence of sulphate-reducers and methanogen under excess sulphate has also previously been revealed for particulate material, with a distinct outer core of sulphate-reducers and an inner core of methanogens (Santegoeds et al., 1999; O'Reilly and Colleran, 2006). The spatial segregation of sulphate-reducers and methanogens is also indicative of the biochemical gradients that can exist (Overmeire et al., 1994). Anoxia is traditionally considered a pre-requisite for the metabolism of these organisms, however sulphate-reducers have cellular mechanisms to cope with oxygen stress (Cypionka, 2000) and active sulphate reduction has been observed in the oxic marine sediments (Jørgensen and Bak, 1991). It is therefore possible that sulphate-reducers produce the metabolic substrates and further reduce the conditions for the more fastidious anaerobic methanogens.

### *Methanogens*

In this study, euryarchaeal populations were identified using 16S rRNA gene analysis from faecal pellets collected from cultured copepods and natural zooplankton populations (Chapter 7). Clusters of 16S rDNA sequences closely related to *Methanogenium*, *Methanobacterium* and *Methanolobus* were identified. The identification of

*Methanogenium* and *Methanobacterium* clones suggests that methanogenesis within the upper water column is also mediated by CO<sub>2</sub>-reducing methanogens and methylotrophy may not be the main pathway of methane production in copepod faecal pellets. The presence of CO<sub>2</sub>-reducing methanogens supports previous suggestions by Marty (1993) of non-methylotrophic methanogen pathways during incubations with zooplankton faecal pellets, although no specific identification was formalized during their study.

### *Methanotrophs*

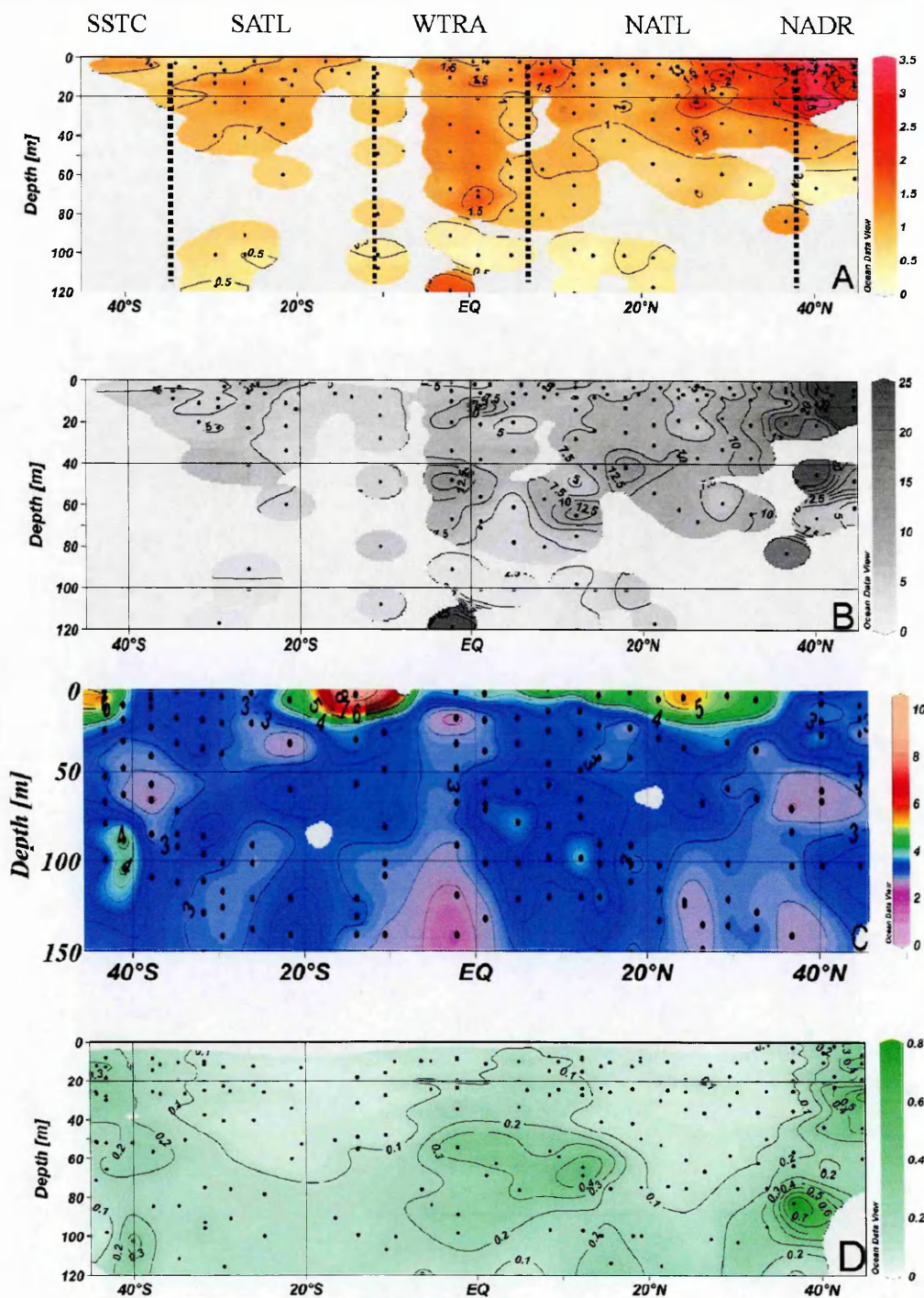
Another potential member of the microbial consortia are the methanotrophs; methane-oxidising bacteria that live in opposing gradients of methane and oxygen. As methane is produced at the centre of anoxic aggregates, it can be oxidised by methanotrophs that simultaneously consume dissolved oxygen. There is relatively little information on the presence of methanotrophs in the upper water column and their existence would support the evidence of methanogenesis in particulate material. Sieburth (1991) reports the presence of *Methylomonas pelagica* and incorporates the methanotroph into the working model of his microbial consortia (Fig 9.3).

It remains to be seen whether methanogens, sulphate-reducers, syntrophic bacteria and methanotrophs can be identified and isolated from pelagic particulate material such as copepod faecal material. The existence of a wider anaerobic food web would strengthen the evidence for microbial methanogenesis in the anoxic micro-niches. Recent technological advances that provide an insight into microbial phylogeny and function will undoubtedly play a role in unravelling the functioning of any existing anaerobic communities. These include micro-autoradiography with CARD-FISH or DNA-based stable-isotope probing which allows substrate uptake (e.g. <sup>13</sup>C-DMSP) by the different

members of the microbial consortia to be tracked. Stable isotope probing has been applied to the study of functionally active methanotroph populations in a range of environments including peat soils (Morris et al., 2002) and aquatic sediments (Lin et al., 2004).

## 9.2 Correlation between DMS, DMSP and methane in the Atlantic Ocean

This Chapter has so far discussed methane and DMS biogeochemistry at the microbial level, highlighting the micro-habitats and the micro-organisms that are involved in this process. Methane and DMS biogeochemical cycling can also be examined at the ecosystem level, *i.e* the pelagic environment. Although water column profiles of DMS, DMSP and methane were not simultaneously analysed in this study, they were jointly measured during the Atlantic Meridional Transect (AMT) programme ([www.amt-uk.org](http://www.amt-uk.org)), cruise number AMT-12. The AMT programme is a time-series of oceanographic stations along a 13,500 km transect in the Atlantic Ocean, crossing major biogeochemical provinces including the Atlantic oligotrophic gyres. The latitudinal plots of DMS+DMSP and methane have kindly been provided by Dr. Bell (Bell, 2006) and Dr. Forster (Forster, 2006) respectively (Fig. 9.4).



**Figure 9.4** Depth contour plots of (a) DMS ( $\text{nmol dm}^{-3}$ ); (b) DMSPp ( $\text{nmol dm}^{-3}$ ); (c) methane ( $\text{nmol dm}^{-3}$ ) and (d) TChl *a* ( $\text{mg m}^{-3}$ ) along AMT-12. Biogeographical provinces are: South Subtropical Convergence (SSTC); South Atlantic Gyre (SATL) Western Tropical Atlantic (WTRA); North Atlantic SubTropical Gyre (NATL); North Atlantic Drift (NADR)

The oligotrophic South Atlantic Gyre and the North Atlantic Sub-Tropical Gyre have less than  $\sim 0.1 \text{ mg m}^{-3}$  TChl *a*. This is typical of oligotrophic waters where low biological productivity and biomass are associated with the nutrient-deplete conditions. In the oligotrophic gyres, methane concentrations are supersaturated in the top 20 m surface waters. Similar supersaturation concentrations were previously observed during longitudinal transects across the Atlantic at  $7^{\circ}30'\text{N}$  and  $4^{\circ}30'\text{S}$  (Oudot, 2002). However, DMS and DMSP concentrations show no similar increase in the oligotrophic gyres. Instead the higher latitude regions of the AMT transect show an increase in DMS and DMSP accompanied by an increase in TChl *a*. The latitudinal trend in DMS concentrations was also apparent in a global DMS database compiled by Kettle et al. (1999). The data from AMT-12 demonstrates that at the biogeochemical provincial scale, an increase in the availability of DMS and DMSP does not result in higher levels of methane. However, whilst this broad trend is evident at the ecosystem scale, it should be noted that the data was not intended to resolve sulphur cycling at the micro-scale and the metabolism of methylated sulphur compounds might well have been occurring.

It is possible that concentrations in DMS(P) and methane concentrations will alter with future climatic-induced changes in ocean structure *e.g.* sea temperature, slowed deep-water formation and water-column stratification (Houghton, 2001). The surface oceans are considered to become increasingly stratified due to global warming with a subsequent effect on nutrient mixing and phytoplankton bloom longevity (Le Quere et al., 2003). It is conceivable that levels of methane at the pycnocline *e.g.* Figure 1.12, will increase due to greater particulate material accumulation as a result of enhanced stratification. Oceanic stratification is also predicted to cause an overall decrease in the global oxygen inventory (Keeling and Garcia, 2002), which would assist the oxygen-sensitive methanogenic

Archaea. The mixing of the surface layer also influences the concentrations of DMS over short timescales (Simó and Pedros-Alio, 1999). This is considered to be partly due to a change in the algal community composition in favour of the DMSP producers such as small flagellates, coccolithophores and dinoflagellates (Simó and Pedros-Alio, 1999).

It remains to be seen whether climatic-induced changes on physical processes and biogeochemical processes will favour increased metabolism of DMS(P) by methanogens and an increased net concentration of methane in oceanic surface waters. A better understanding of the process will be facilitated by a greater temporal resolution of the water-column methane profiles. This can be provided by *in situ* methane sensors, which are currently deployed to investigate gas seeps and deep-ocean waters (Brewer et al., 2004; Marinaro et al., 2004). In addition, latitudinal transects in microbial community structure to investigate possible changes at sites of elevated methane. Furthermore, the presence of other reduced gases such as hydrogen sulphide would provide independent corroboration of micro-zones that could facilitate methanogenesis.

It should also be noted that an explanation for the observed methane profiles in Figure 9.4 might not solely be dependent upon methanogenic Archaea in anoxic micro-niches. It is possible that a non-microbial source of methane exists, as mentioned in Section 1.2.5.

Terrestrial plants were recently discovered to emit methane (Keppler et al., 2006) and it has previously been suggested that there is an algal production of methane (Scranton and Brewer, 1977). In addition, the effect of methane oxidation needs to be taken into consideration as an accumulation of methane could occur due to decreased oxidative processes. The relatively few measurements of aerobic methane oxidation suggest that turnover rates in the water column are very low (Scranton and Brewer, 1978). It has



recently been highlighted that light intensity can have an inhibitory effect on methane oxidation in the surface waters of fresh-water lakes (Murase et al., 2005), and it is possible this also occurs in oceanic surface waters.

### 9.3 From the microbial scale to the global scale

One intriguing aspect of this study is unravelling how the metabolic by-products of micro-organisms affect the Earth at the global scale. For example, microbes influence the Earth's atmospheric chemistry through oxygenic photosynthesis, nitrogen fixation and carbon fixation (Newman and Banfield, 2006). Furthermore, before the evolution of atmospheric oxygen, it is considered likely that methane was abundant in the anoxic atmosphere due to methanogenic Archaea (Pavlov et al., 2000). The interaction between biota and climate is also considered by many researchers to be a two-way process and a number of feedback mechanisms have been proposed *e.g.* the CLAW hypothesis (Section 1.1.5).

Although microbial activity influences the Earth's climate, the appropriate micro-organisms have not always been represented in biogeochemical modelling. More recently marine biogeochemical models have incorporated pelagic functional groups. The term 'pelagic functional group' refers to groups of organisms that mediate specific chemical reactions in the oceans *e.g.* nitrogen fixers, silica producers, calcifiers and DMS producers (as listed by Le Quere et al. (2005), and Hood et al. (2006)). The DMS producers refer to the phytoplankton that produce DMSP, as identified in Chapter 3. However, the inclusion of DMS producers in models does not indicate that our knowledge of the process is complete. The reason why algae produce DMSP and DMSO remains unclear and the correlation between DMSP and DMS production is not straightforward as observed in Chapter 4, where algae, bacteria and higher trophic organisms all played a role in DMS

production. It also remains to be seen whether DMSO will add an extra layer of complexity of the cycling of methylated sulphur compounds in the upper ocean, or whether it will resolve some of the uncertainty surrounding DMSP and DMS interactions.

This thesis highlighted that DMS is not the only compound produced by the bacterial metabolism of DMSP and DMSO. It is possible that within anaerobic micro-niches, methanogens can indirectly utilise DMSP and DMSO. The consequence of the metabolism by methanogens can also be considered at a global scale as the marine emissions of DMS and methane and their subsequent effects on the Earth's atmosphere can be compared using the IPCC report (2001) (Table 9.1). It should be noted that the comparison of methane and DMS emissions from the oceans is based on their net concentrations in the surface oceans and does not reflect how much methane is being derived from either DMSP or DMS.

The relative impact of methane and DMS on the Earth's climate is compared on the basis of 'radiative forcing' (Table 9.1). Radiative forcing as defined by the IPCC is 'the change in net irradiance at the tropopause after allowing for stratospheric temperatures to readjust to radiative equilibrium' and it is a useful tool to estimate the relative climatic impacts due to radiatively induced perturbations (*e.g.* DMS and methane). The IPCC stipulate that the negative and positive global mean forcings cannot be added up to indicate an average in the global climate impact. This is because even in the event of zero net radiative forcing, spatial and temporal influences can cause localised forcing.

Keeping this IPCC stipulation in mind, the emissions of DMS and methane from the marine environment are summarised in Table 9.1. This value is then used as a percentage

to calculate the relative quantity of radiative forcing that is associated with these emissions respectively.

**Table 9.1** Comparison of the atmospheric properties, and the emissions of methane and dimethylsulphide from the marine environment. <sup>1</sup>Kloster et al. (2006).

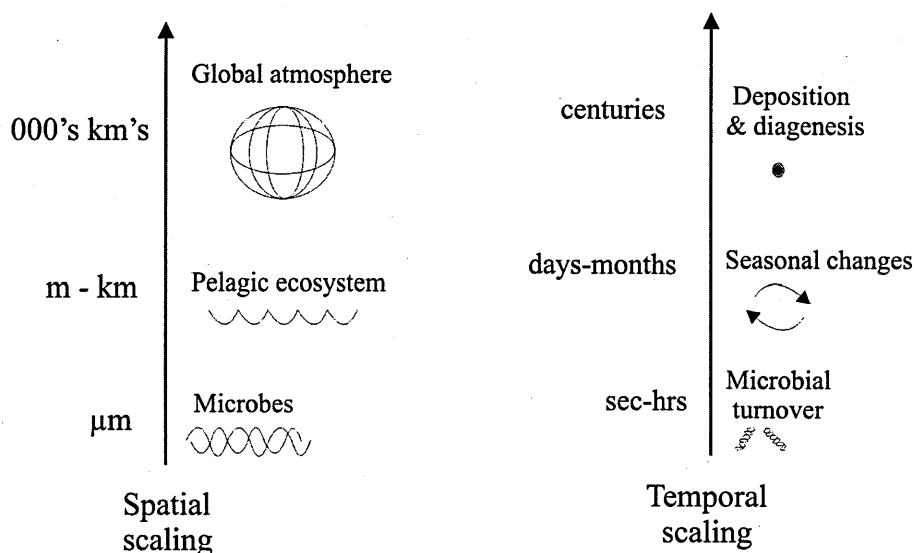
	Methane	Dimethylsulphide
Atmospheric lifetime	25 years	0.5 – 1 day
Atmospheric distribution	Homogeneous	Heterogeneous
Atmospheric effect: Direct	Absorbs long wave radiation	Scatters & absorbs infrared radiation
Indirect	Atmospheric chemistry	Cloud properties
Radiative forcing: Direct (W m <sup>-2</sup> )	0.38	-0.26 to -0.82
Indirect	0.12	-0.3 to -1.8
Total	0.5	-1.59
Marine emissions (Tg/yr)	10-15	24
% contribution to respective atmospheric loading	2 - 2.5%	27% (sulphate budget <sup>1</sup> )
Quantity of radiative forcing associated with emissions from marine environment (Wm <sup>-2</sup> )	0.0114	-0.4293

It is evident in Table 9.1 that the radiative forcing associated with methane being emitted from the oceans is much smaller than DMS. For the Earth to be in equilibrium with regards to its radiative forcing, the sum of the values should balance over the appropriate temporal and spatial periods. Therefore to account for the discrepancy between the marine emissions of DMS and methane, other sources of methane and DMS, and other radiative agents need to be taken into consideration, as detailed in the IPCC (2001) report. It is also clear that changes in the marine emissions of DMS can alter the Earth's radiative balance and affect global warming (Charlson et al., 1987). For example, a doubling in atmospheric CO<sub>2</sub> concentrations is associated with a small increase (3 %) in the global DMS flux which is estimated to increase the radiative effect resulting from the DMS-induced change in cloud albedo by -0.05 Wm<sup>-2</sup> (Bopp et al., 2003).

One of the major difficulties in considering the link between methane and DMS in the open ocean is that methanogenesis in the upper ocean is not solely dependent upon a supply of methylated sulphur compounds. Ambient oxygen concentrations, interactions with sulphate-reducing bacteria, and oxidative processes will also determine the quantity of methane produced and emitted from the oceans. A more clear-cut example of the interaction between methane production and the sulphur cycle has been demonstrated in terrestrial wetlands (Gauci et al., 2004). The deposition of sulphate associated with industrial combustion of fossil fuels has the potential to divert substrate flow away from methanogenesis to sulphate-reduction in the peatlands and thereby inhibit the methane flux to the atmosphere (Schimel, 2001). Gauci et al. (2004) estimates that sulphur pollution reduces methane emissions from the peatlands by 25 Tg (or 8 %).

The production of trace gases DMS and methane, as demonstrated in this study, is just one of the many ways micro-organisms affect the chemistry and physical properties of their surroundings. These processes are complicated by interactions between the microbial organisms and biogeochemical cycles. This is exemplified by the inhibition of methanogenesis in terrestrial wetlands by sulphate reduction, as reported by Gauci et al. (2004), and the metabolism of DMSP by methanogenic Archaea in faecal pellets within the upper water column. The identification of appropriate microbial community dynamics and physiology that affect the global scale processes is critical to understanding biogeochemical systems. Figure 9.5 depicts the different spatial scales discussed in this Chapter, ranging from the micro- (microbial); meso- (pelagic ecosystem) and mega-scale (the global atmosphere). A consideration of DMS and methane interactions at these different scales was a useful way to illustrate the findings of this research project. It also helped to identify areas where further work is required to resolve our understanding of

DMS and methane dynamics. As future research continues to investigate global climate change and the role of microbial communities in ecosystem functioning, it is anticipated that the connections between these different spatial and temporal scales will strengthen.



**Figure 9.5** Spectrum of spatial and temporal scales to highlight the relevant biogeochemical impacts of microbial activity.

#### 9.4 Future work

The discussion on the interaction between DMS and methane in this Chapter has considered the combination of different components of microbial biogeochemistry and oceanography. However each of these components *e.g.* DMS/DMSP cycling, DMSO, biogas measurements and microbial community composition, are also important in their own right and recommendations for further work are made with reference to specific sections.

This study analysed the production of DMSO by different phytoplankton species and compared the cellular concentrations with DMSP values. Future work should extend

measurements to algal species beyond the three major phylogenetic groups analysed in Chapter 3. Measurements of DMSO (and DMSP) should ideally be normalised to cellular carbon or pigments and other appropriate biomarkers. The factors that affecting cellular DMSP:DMSO ratios are discussed in Chapter 3 and further experiments should aim to elucidate these factors. For example, how do oxidative stress mechanisms *e.g.* solar radiation, nutrient depletion, affect the DMSP:DMSO ratio?

The zooplankton grazing experiments in Chapter 4 revealed that DMS is not produced in the presence of antibiotics. This result reinforces the notion that microbes play a central role in the cycling of DMSP and DMS. The relationship between microbial systems *e.g.* growth rate, species composition, and the dynamics of DMS is likely to prove fundamental to the understanding of DMS in the global oceans. In addition, the importance of DMSO in the DMSP/DMS cycling needs to be determined at the appropriate temporal and spatial scale. This study identified that within sinking particles a net production of DMSO is observed. This needs to be placed within a wider context of under what conditions in the marine environment does DMSO represent a net source for DMS.

This study investigated DMS and methane dynamics in the Scottish coastal environment although due to technical constraints, it was not possible to conduct in-depth methane measurements in the water column. After reflection of the AMT dataset in section 9.2, it is considered that to look at the interaction between methane and DMS on a detailed profile of the water column, a greater resolution of the dataset is required. This could be provided by the use of *in situ* sensors, as discussed in Section 9.2.

To investigate the potential for methanogenesis in particulate material this study incubating sample material collected in the sediment trap under anaerobic conditions. A more appropriate methodology would take into consideration the ambient oxygenated water column and therefore experiments should also be conducted under aerobic conditions. One possible methodology is to use labelled-compounds *e.g.*  $^{14}\text{C}$ - $\text{CO}_2$  or  $^{14}\text{C}$ -DMS, followed by fingerprinting of the microbial community such as stable isotope probing to identify the active individuals. However such a more directed approach should not neglect the potential for non-microbial source of methane and an algal source of methane should also be investigated further.

One of the most interesting findings of this thesis was the characterisation of the methanogen community by 16S rDNA analysis. Samples continue to be collected from different species of copepod maintained in culture and faecal pellets have been collected from natural communities of zooplankton at two geographical regions, the mid-Atlantic and the oligotrophic central North Pacific. It remains to be seen whether the same species or a wider diversity of methanogenic archaea is observed in these samples. Future molecular analysis could also target the *mcrA* gene as a functional gene marker as demonstrated for other methanogenic habitats.

## 9.5 Conclusion

This thesis investigated the interaction between DMSP, DMSO and DMS and methane within micro-environments in the upper water column. Within the pelagic environment, a number of DMSP and DMSO 'hotspots' were identified, which represented microenvironments with elevated concentrations of methylated sulphur compounds *e.g.* algal cells, faecal pellets and other particulate material. The production of DMSO by phytoplankton and the release of DMSO from algal cells into the ambient water column are not typically considered in the classical methylated sulphur cycles. It is suggested that DMSO plays a much greater role in the cycling of DMSP and DMS than currently anticipated. Within microenvironments, the potential depletion of oxygen could lead to the metabolism of DMSP, DMSO and DMS by methanogens either indirectly or directly. The methane produced as a metabolic by-product would contribute to the supersaturation observed in the oceanic surface waters. The incubation of particulate material with different sulphur compounds and inhibitors, together with the characterisation of the methanogen community by 16S rDNA analysis, suggests that a wider consortium of bacteria facilitate this process. It remains to be seen whether the production of methane as outlined in this discussion exists *in situ* within the upper water column, however the results from this thesis provide a realistic mechanism for its occurrence.





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